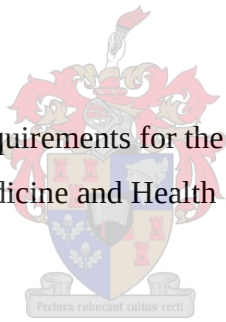
The carriage of antibiotic resistant Gram-negative organisms in children in the Cape Town community and the impact of antibiotic exposure on the development of resistance

(a TB-CHAMP sub-study)

by
Remous Ocloo



Thesis presented in fulfilment of the requirements for the degree of Master of Science in Medical Microbiology in the Faculty of Medicine and Health Sciences at Stellenbosch University

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Declaration

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Abstract

Introduction

Antibiotic resistance has become a major issue across the globe and the situation is worsening in low- and middle-income countries. In sub-Saharan Africa and the world at large, antibiotic resistance research is localized and focused on hospitalized individuals. There is, therefore, little or no data on antibiotic resistance in the community; especially in children. This study described the carriage of resistant isolates in children in Cape Town and investigated the effects of antibiotic exposure on the development of resistance in stool using an *in-vitro* model.

Materials and Methods

Stool samples from fifty participants of the Tuberculosis Child Multidrug-resistant Preventive Therapy Trial (TB-CHAMP) were cultured onto McConkey agar (MCC) with the addition of ertapenem and cefpodoxime discs to select for carbapenem and cephalosporin-resistant and susceptible *E. coli* and *Klebsiella* isolates. Antibiotic susceptibility testing was performed using Kirby Bauer disk diffusion. Carbapenem-, quinolone- and cephalosporin-resistance genes were detected by PCR and resistance-conferring mutations were detected using Sanger sequencing.

Ten stool samples were exposed to two sub-clinical concentrations of amoxicillin, ciprofloxacin and colistin for 48 hours, whereafter they were plated onto MCC with the addition of various antibiotic discs (amoxicillin, ertapenem, ciprofloxacin, colistin, cefotaxime and nalidixic acid). The impact of antibiotic exposure on the development of resistance was assessed by enumeration of presumptive resistant *E. coli* and *Klebsiella* colonies within the zones of inhibition around the antibiotic discs.

Results

Twenty-one (42%) of the participants were colonized by quinolone-resistant isolates and 18 (36%) by cephalosporin-resistant isolates (predicted ESBL-producing organisms). Of the 21 quinolone-resistant *E. coli* isolates, 5 (24%) harbored *qnrS* while of the 6 quinolone-resistant *Klebsiella* isolates, 4 (67%) had *qnrB.* The most common quinolone resistance mutations were S83L in *gyr*A and S80I, A141V and S129A in *parC*. *bla*CTX-M was the only ESBL gene detected. All of the *bla*SHV and *bla*TEM genes detected were β-lactamases without extended

spectrum activity. One of the participants was colonized by a carbapenem resistant *Klebsiella* isolate, which carried the *bla*NDM carbapenemase gene.

Exposure of the stool samples to ciprofloxacin selected for resistant bacteria, however exposure to amoxicillin and colistin did not.

Conclusion

Children in Cape Town are frequently colonized by resistant bacteria and are at risk of becoming infected by these resistant organisms. The presence of plasmid-mediated resistance genes is concerning because they can be transferred between bacteria of the same and different species. There is also a need to further investigate what might be driving the high prevalence of quinolone resistance in the community. This study is the first to report the carriage of carbapenemase resistant bacteria in healthy children in South Africa.

Although the *in-vitro* antibiotic exposure model was crude, the approach provides some evidence for the development of resistance during exposure to sub-clinical concentrations of antibiotics (especially ciprofloxacin); and notably, to agents other than those to which the sample had been exposed. This highlights the need for further investigations into the impact of sublethal antibiotic concentrations on the selection of resistance.

Opsomming

Inleiding

Antibiotiese weerstand is ‘n wêreldwye probleem, en die situasie is besig om in lae- en middelvlakinkomstelande te versleg. In sub-Sahara Afrika, en ook die res van die wêreld, fokus navorsing oor antibiotiese weerstand grootliks op gehospitaliseerde individue en is meestal gelokaliseerd. Daar is dus min tot geen data oor antibiotiese weerstand in die gemeenskap nie; veral in kinders. Hierdie studie beskryf die verspreiding van weerstandbiedende bakteriële isolate in Kaapstad en het die effek van antibiotika-blootstelling op ontwikkeling van weerstand in stoelmonsters met ‘n *in-vitro* model ondersoek.

Metodes

Stoelmonsters vanaf vyftig deelnemers van die “Tuberculosis Child Multidrug-resistant Preventive Therapy Trial” (TB-CHAMP) is op McConkey agar (MCC) met ertapenem en cefpodoksiem skyfies geweek, om karbapenem- en kefalosporien-weerstandbiendende en -vatbare *E. coli* en *Klebsiella* isolate te selekteer. Antibiotiese vatbaarheidstoetse is deur Kirby Bauer skyfiediffusie uitgevoer. Karbapenem, kinoloon en kefalosporien-weerstandsgewende mutaties is met Sanger DNA-volgordebepaling bespeur.

Tien stoelmonsters was vir 48 uur aan twee subkliniese konsentrasies van amoksisillien, siprofloksasien en colistin blootgestel, waarna hulle op MCC gekweek is met verskeie antibiotika skyfies (amoksisillien, ertapenem, siprofloksasien, colistin, kefotaksiem en nalidiksiensuur). *Escherichia coli* en *Klebsiella* kolonies binne die inhibisie-zones rondom die antibiotika skyfies is getel om die impak van antibiotika-blootstelling op die ontwikkeling van weerstand te bepaal.

Resultate

Een-en-twintig (42%) van die deelnemers was met kinoloon-weerstandige isolate gekoloniseer en 18 (36%) deur kefalosporien-weerstandige isolate (voorspel om “Extended spectrum beta-lactamase”-produserend (ESBL) te wees). Van die 21 kinoloon-weerstandige *E. coli* isolate, het 5 (24%) *qnrS* gene besit, terwyl 4 (67%) van die kinoloon-weerstandige *Klebsiella* isolate positief getoets het vir *qnrB.* Die algemeenste kinoloon weerstandgewende mutaties was S83L in

*gyr*A en S80I, A141V en S129A in *parC*. *bla*CTX-M was die algemeenste ESBL geen wat geïdentifiseer is. Geen van die geïdentifiseerde *bla*SHV en *bla*TEM gene was ESBL-produserend nie. Een deelnemer was met ‘n karbapenem-weerstandbiedende *Klebsiella* isolaat met ‘n *bla*NDM karbapenemase geen gekoloniseer.
In die stoelmonsters het blootstelling aan siprofloksasien, eerder as amoksisillien en colistin, vir weerstanbiedende bakterieë geselekteer.

Gevolgtrekking

Kinders in Kaapstad word gereeld deur weerstandbiedende bakterieë gekoloniseer en beloop die risiko om deur hierdie organismes geïnfekteer te word. Die teenwoordigheid van plasmied-bemiddelde weerstandsgene is daarom kommerwekkend, aangesien hulle tussen bakterieë van dieselfde en verskillende spesies oorgedra kan word. Dit is dus nodig om verdere ondersoek te doen om te bepaal wat die hoë vlak van kinoloon-weerstand in die gemeenskap veroorsaak. Hierdie studie bevat die eerste beskrywing van die verspreiding van karbapenem weerstandbiedende bakterieë in gesonde kinders in Suid-Afrika.

Alhoewel die *in-vitro* antibiotika-blootstelling model nie gesofistikeerd was nie, het hierdie benadering bewys gegee van die ontwikkeling van weerstand tydens blootstelling aan subkliniese konsentrasies van antibiotika (veral siprofloksasien); merkwaardig ook aan ander middels waaraan die monsters nie blootgestel was nie. Dit wys dat verdere ondersoek ingestel moet word om die impak van subkliniese antibiotika konsentrasies op die seleksie van weerstand te bepaal.

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List of Abbreviations

AMR	Antibiotic Resistance
API	Analytical Profile Index
AST	Antibiotic Susceptibility Testing
BRICS	Brazil, Russia, India, China, South Africa
BSI	Bloodstream Infections
CA	Community-Acquired
CDC	Centers for Disease Control and Prevention
CDDEP	Center for Disease Dynamics, Economics and Policy
CLSI	Clinical and Laboratory Standards Institute
CPE	Carbapenemase-Producing Enterobacteriaceae
CRE	Carbapenem-Resistant Enterobacteriaceae
DNA	Deoxyribose Nucleic Acid
DTTC	Desmond Tutu TB Centre
EARS-NET	European Antibiotic Resistance Surveillance Network
ECDC	European Centers for Disease Control and Prevention
ESBL	Extended Spectrum β-Lactamase
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp.
FAO	Food and Agriculture Organization
FQN	Fluoroquinolone
GERMS-SA	Group for Enteric, Respiratory and Meningeal disease surveillance in South Africa
GIT	Gastrointestinal Tract
HA	Hospital-Acquired
HREC	Health Research Ethics Committee
INFORM	International Network for Optimal Resistance Monitoring
INH	Isoniazid

KPC	*Klebsiella pneumoniae* Carbapenemase
MDR	Multidrug-Resistant
MIC	Minimum Inhibition Concentration
MLST	Multi-Locus Sequence Typing
NAC	No Antibiotic Control
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
PBP	Penicillin-binding Proteins
PCR	Polymerase Chain Reaction
PEARLS	Pan-European Antimicrobial Resistance using Local Surveillance
PFGE	Pulsed-Field Gel Electrophoresis
PMQR	Plasmid-mediated Quinolone Resistance
RAPD	Random Amplified Polymorphic DNA
REP	Repetitive Extragenic Palindromic
RFLP	Restriction Fragment Length Polymorphism
SAASP	South African Antibiotic Stewardship
SASCM	South African Society of Clinical Microbiology
SMART	Study for Monitoring Antimicrobial Resistance Trends
SOP	Standard Operating Procedure
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TB	Tuberculosis
TBA	Tryptose Blood Agar
TB-CHAMP	Tuberculosis Child Multidrug-resistant Preventive Therapy Trial
TRUSB	Transrectal Ultrasound-Guided Needle Biopsy
UK	United Kingdom
UPMGA	Unweighted Pair Group Method with Arithmetic
US	United States
USA	United States of America
USD	United State Dollars

UTI	Urinary Tract Infections
VIM	Verona Integron-encoded Metallo-β-lactamase
WHO	World Health Organizations

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Appendix

Chapter 1 : Literature Review

1.1 The Global Problem of Antibiotic Resistance

The discovery of antibiotics in the early 1930s was considered the beginning of a "golden age" in the history of medical practice. Antibiotics made a significant impact on public health, allowing infections to be more easily treated and resulting in lives being saved (Grundmann, 2014; Lee *et al.*, 2013; Smith & Read, 2016). Nonetheless, this "golden age" is soon to end, since the 21st century has encountered antibiotic resistance, and society is now faced with what is being referred to as a return to the "pre-antibiotic era", where antibiotic use is less effective. The reduced efficacy of antibiotics has become a threat not only in nosocomial settings but also in the community. The issue of antibiotic resistance does not affect only patients but also politicians, health insurance companies and global health donors, all of whom play a key role in global health (Grundmann, 2014). The anticipation that a novel antibiotic will be discovered to combat antibiotic resistance is diminishing, especially for Gram-negative bacteria (Lee, Cho, Jeong & Lee, 2013).

In 2013, the Centers for Disease Control and Prevention (CDC) released a list of the top 18 antibiotic-resistant microorganisms which require serious attention; this included carbapenem-resistant Enterobacteriaceae (CRE) and extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae (CDC, 2013). European CDC reports identify 3rd generation cephalosporin resistance and multidrug resistance to be frequent in *Escherichia coli* and *Klebsiella pneumoniae* (ECDC, 2015).

The 2013 CDC report shows that in the United States (US) alone, 2 million individuals acquire antibiotic-resistant infections annually with 23 000 deaths as a result (CDC, 2013). The US spends $21 000-$34 000 million per year on resistance while Europe spends €1 500 million per year (Roca *et al.*, 2015). Generally, the prevalence and growth rate of antibiotic resistance is 17% and 7% respectively in the US (Zhang *et al.*, 2006). Without any global action to mitigate and fight antibiotic resistance, there would be 10 million deaths across the globe annually due to antibiotic resistance infections by 2050. This means that the world will generate approximately 8

trillion United States Dollar (USD) less each year by 2050 and the world will lose 100 trillion USD within the next 35 years (O'Neill, 2016).

There are several factors that drive the production of resistance, including the increase in ESBLs in Enterobacteriaceae, but high intake of antibiotics is a major factor (Storberg, 2014). Antibiotic consumption has increased significantly in Brazil, Russia, India, China, South Africa (BRICS) countries, and a similar increase was observed in West Africa. Seventy-six percent of the total increase in global antibiotic consumption from 2000-2010 was associated with BRICS countries. However, only 33% of the total increase in the world's population occurred in BRICS countries between those years. In BRICS countries, 23% of the increase in the retail sales volume was associated with India and 57% of the increase in clinical settings was in China (Van Boeckel *et al.*, 2014). Over 90% of antibiotics used in clinical settings in Europe are prescribed to outpatients (Bell *et al.*, 2014).

1.2 Gram-Negative Bacteria (GNB) and Infections

The Enterobacteriaceae is a family of Gram-negative bacilli that can cause both nosocomial and community-acquired (CA) infections. Of the Enterobacteriaceae, *E. coli* and *K. pneumoniae* are among the most commonly isolated pathogens (Iredell *et al.*, 2016).

While *E. coli* is a common enteric commensal, it is also the most frequently isolated Gram-negative bacterium in clinical samples and has the ability to cause a range of clinical infections, such as pyelonephritis and gastroenteritis (Rezazadeh *et al.*, 2016). *E. coli* is the commonest cause of urinary tract infections (UTI), and it has been estimated that approximately 150 million individuals across the globe acquire UTIs annually (Fasugba *et al.*, 2015). *E. coli* is the leading cause of acute diarrhea in most developing countries and it is also associated with enteritis in children less than 5 years (Bii *et al.*, 2005). Bloodstream infections (BSI) are the most common hospital-acquired (HA) infection caused by *E. coli* (Chmielarczyk *et al.*, 2015). Thirty-two percent of bacteremias due to *E. coli* in Auckland (Williamson *et al.*, 2013), Canada (Laupland *et al.*, 2008) and Minnesota (Al-Hasan *et al.*, 2009) were health-care associated.

K. pneumoniae is an opportunistic pathogen associated with both nosocomial and community-acquired infections (Chung, 2016). *K. pneumoniae* has the ability to cause several infections including bloodstream infections, pneumonia, skin structure infections and UTIs (Cprek & Gallagher, 2015). In addition, *K. pneumoniae* can also cause septicemia, liver abscesses and meningitis (Decré *et al.*, 2011). Epidemiological studies have shown that colonization of the gastrointestinal tract (GIT) occurs prior to most *K. pneumoniae* infections (Struve & Krogfelt, 2004). From 1996-1997 in the USA, 68 cases of CA bloodstream infections were reported in two hospitals and 43% were due to *K. pneumoniae*. Forty out of 116 cases of CA bloodstream infections in Johannesburg, South Africa were due to *K. pneumoniae* (Ko *et al.*, 2002). Of 864 cases of pediatric BSI reported in Cape Town of which 404 were HA and 460 CA , nearly 18% were due to *K. pneumoniae* from 2008-2013 (Dramowski *et al.*, 2015).

1.3 Antibiotics and Resistance Mechanisms in Enterobacteriaceae

Infections with Enterobacteriaceae can be treated using a range of antibiotics (Penesyan *et al.*, 2015). Different classes have different mechanisms of action, as illustrated in Figure 1.1. Third generation cephalosporins, aminoglycosides, polymyxins and fluoroquinolones (ciprofloxacin and levofloxacin) have proved to be efficacious against Enterobacteriaceae infections.

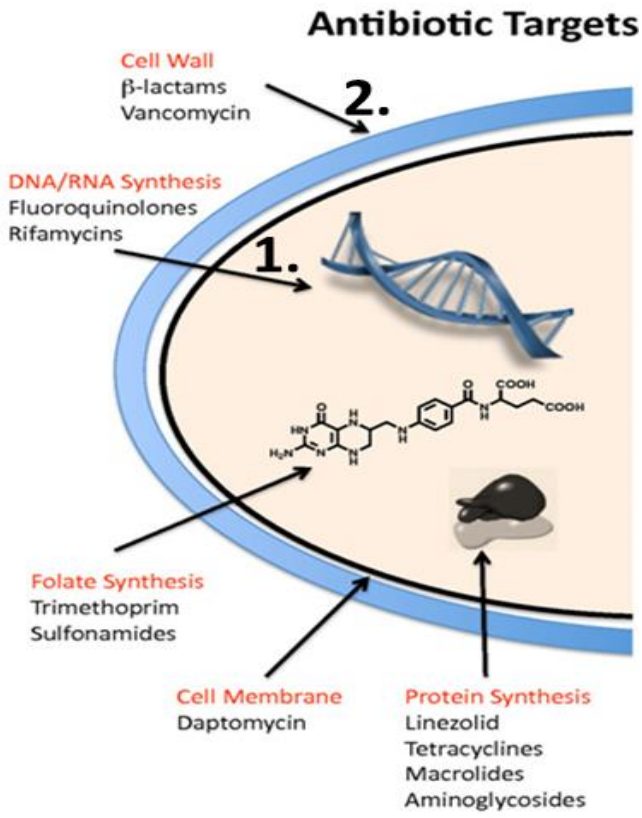


Figure 1.1. Various classes of antibiotics and their modes of action. The target site of antibiotics depends on their class. (1) Fluoroquinolones target DNA and RNA synthesis (2) β-lactams target the bacterium cell wall.

(Adapted from:https://upload.wikimedia.org/wikipedia/commons/6/61/Antibiotic_resistance_mechanisms.jpg)

The increase and inappropriate use of antibiotics has selected mutant bacteria which develop resistance to antibiotics (Penesyan *et al.*, 2015). In recent years Enterobacteriaceae have been observed to be resistant to third-generation cephalosporins, carbapenems, aminoglycosides, polymyxins, and fluoroquinolones (Kocsis and Szabó, 2013). The focus of this research project is on resistance to β-lactams and fluoroquinolones, and the following sections will describe the mechanisms of action and resistance to these agents in more detail.

1.4 β-lactams and β-lactamases

β-lactam antibiotics are a commonly used class of antibiotic. They are broad-spectrum antimicrobials which include all antibiotics with a β-lactam ring in their molecular structure. Examples include penicillins, cephalosporins, monobactams and carbapenems (Lakshmi *et al.*, 2014). The cephalosporin class of antibiotics includes ceftazidime, cefepime, cefuroxime, cefpodoxime, and oxyimino-monobactam (Ghafourian *et al.*, 2014). Cephalosporins are relatively broad-spectrum antibiotics, with the more recent generations showing increasing activity against Gram-negative organisms. Carbapenems are broad spectrum agents, active against a wide range of Gram-positive and Gram-negative bacteria, and include ertapenem, imipenem, meropenem and doripenem. β-lactams exert their bactericidal effect because of their ability to inhibit penicillin-binding proteins (PBP) and thus inhibit cell wall synthesis (Kong *et al.*, 2010).

While resistance to β-lactam antibiotics can arise through a variety of mechanisms (Figure 1.2), production of β-lactamases is the most common mechanism used by Enterobacteriaceae to confer resistance to these agents. These enzymes inactivate β-lactam antibiotics by hydrolysis; they bind to the β-lactam ring of the antibiotic, thereby deactivating the molecule. Examples of β-lactamases include *bla*AmpC, ESBLs and carbapenemases (Lakshmi *et al.*, 2014). β-lactamases have been classified by Ambler based on peptide sequence analogy and phenotypic characteristics (Ambler, 1980), while the Bush classification is based on functional and molecular groups (Bush *et al.*, 1995).

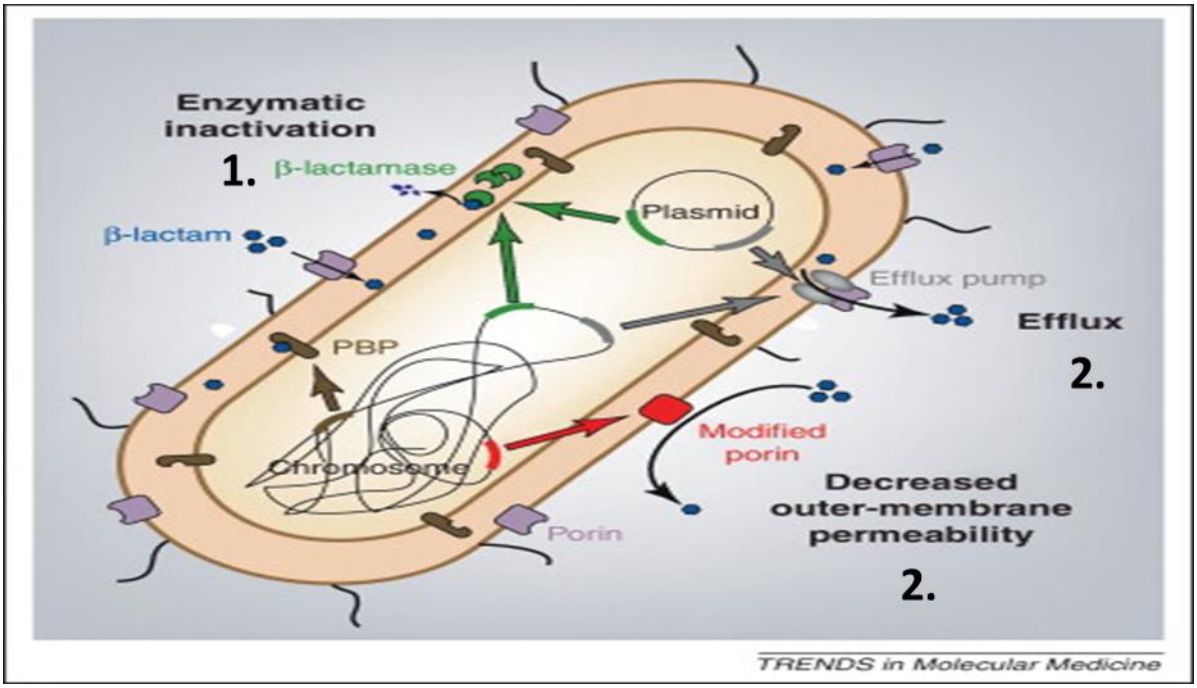


Figure 1.2: **β-lactam resistance mechanisms.** The basic mechanisms of β-lactam resistance in Enterobacteriaceae include (1) Enzyme inactivation of β-lactam by hydrolysis; these enzymes are either chromosome or plasmid-mediated. (2) Efflux pumps and loss of porins also help the bacterium to reduce accumulation of drugs. **(Adapted from: Nordmann *et al.* 2012**).

1.4.1 blaAmpC β-lactamase

According to the Ambler classification of β-lactamases, *bla*AmpCs are classified in class C, while the functional classification scheme of Bush *et al* puts them in group 1. *bla*AmpC has a serine residue at its active site.

*bla*AmpC β-lactamases are clinically significant cephalosporinases, which are chromosomally-mediated in certain genera of the Enterobacteriaceae (Jacoby, 2009). In most Enterobacteriaceae, *bla*AmpC genes are expressed at low-levels and are inducible on exposure to β-lactams. Mutations in the *bla*AmpC regulatory regions can also lead to constitutive hyperproduction of the enzyme (Tang *et al.*, 2014). *bla*AmpC β-lactamases hydrolyze both narrow and broad-spectrum cephalosporins (Marsik & Nambiar, 2011). *bla*AmpC has been reported all over Africa in both community and hospital acquired infections (Storberg, 2014). *bla*AmpC was always

known to be chromosomally-mediated, however recently plasmid-mediated *bla*AmpC has been reported but is less common (Tanushree Barua et al. 2013)

1.4.2 Extended-spectrum β-lactamases (ESBLs)

Extended-spectrum β-lactamases are enzymes capable of hydrolyzing narrow and broad-spectrum cephalosporins. ESBLs most often are not active against carbapenems (Paterson & Bonomo, 2005). The commonest types of ESBL are those in the *bla*TEM-, *bla*SHV-, and *bla*CTX-M families (Kocsis & Szabó, 2013).

1.4.2.1 blaTEM β-lactamase

*bla*TEM β-lactamases are categorized by Bush *et al* under the functional group 2b while Ambler's classification lists them as class A β-lactamases (Ambler, 1980; Bush *et al.*, 1995). *bla*TEM enzymes were originally isolated from Enterobacteriaceae (Monstein et al. 2007) and were first reported in Greece in an *E. coli* isolate (Zaniani et al. 2012). The first plasmid-mediated *bla*TEM appeared in the 1960s (Chong, 2011).

More than 130 *bla*TEM-types are known, but not all have extended-spectrum β-lactamase activity (Chong, 2011). *bla*TEM-1, *bla*TEM-2, and *bla*TEM-13 are narrow spectrum β-lactamases, not considered ESBLs (Paterson & Bonomo, 2005). *bla*TEM-1 and *bla*TEM-2 are progenitors, while *bla*TEMs with extended spectrum activity have a range of amino acid substitutions (Jacoby & Munoz-price, 2005). Most *bla*TEM ESBLs have a single amino acid substitution at their active site; this occurs most commonly at positions 104, 164, 238, and 240 (Jacoby & Munoz-price, 2005). *bla*TEM genes are plasmid-mediated.

Novel *bla*TEM β-lactamases have been isolated that are able to hydrolyze third-generation cephalosporins and show resistance to β-lactamase inhibitors. These *bla*TEM β-lactamases are also known as inhibitor resistant β-lactamases because they can reduce the susceptibility of organisms to clavulanic acid, sulbactam, and tazobactam combinations (Jacoby & Munoz-price,

2005; Paterson & Bonomo, 2005). The main producer of *bla*TEM is *E. coli* (Jacoby & Munoz-price, 2005).

1.4.2.2 blaSHV β-lactamase

Extended-spectrum *bla*SHV β-lactamases belong to functional group 2be and have also been allocated to subclass A1 of serine β-lactamases (Liakopoulos *et al.*, 2016). Not all *bla*SHVs have extended spectrum activity; *bla*SHV-1, *bla*SHV-11, and *bla*SHV-26 lack extended spectrum activity (Jiang *et al.*, 2012). The plasmid-mediated *bla*SHV β-lactamases are derived from a chromosomal *bla*SHV-1 "parent" which is predominantly present in *K. pneumoniae. bla*SHV-1 shares 68% of its amino acids with *bla*TEM-1 and they have a similar structure (Jacoby & Munoz-price, 2005). *bla*SHV β-lactamases with extended spectrum activity also have amino acid substitutions at their active site, usually at positions 238 or 240 (Castanheira *et al.*, 2013). An *bla*SHV ESBL was first identified in *E. coli* in the 1970s (Liakopoulos *et al.*, 2016) and in *K. pneumoniae* in the 1980s. More than 50 *bla*SHV-types are currently known (Chong, 2011). One worth noting is *bla*SHV-27, which has been identified on different plasmids in *E. coli* and *K. pneumoniae.* (Chong, 2011).

*bla*SHV has been found outside of the normal clinical hosts *E. coli* and *K. pneumoniae* and has been reported in other Enterobacteriaceae (Liakopoulos et al. 2016). Horizontal gene transfer of plasmid-mediated *bla*SHV between non-related species has also been documented (Garza-Ramos *et al.*, 2007). Some of the *bla*SHV variants co-exist with other resistance genes such as *bla*CTX-M and *bla*VIM (Liakopoulos et al. 2016).

Enterobacteriaceae producing *bla*SHV ESBLs are mostly resistant to oxyimino β-lactams (cefotaxime, ceftazidime) and monobactams (aztreonam) but may be susceptible to carbapenems and can be inhibited by β-lactamase inhibitors (Venezia *et al.*, 1995).

1.4.2.3 blaCTX-M β-lactamase

The *bla*CTX-M β-lactamases got their name from "cefotaximase" activity, which is a distinctive characteristic of these enzymes. This unique ability is attributed to a mobile electrostatic feature which enables them to track cefotaxime to their binding sites and hydrolyze it (Cantón & Coque, 2006). *bla*CTX-M was first detected in Germany and Argentina in 1989, and since then has disseminated worldwide. There are different clones of *bla*CTX-M, the most prevalent include *bla*CTX-M-9, *bla*CTX-M-14, and *bla*CTX-M-15. *bla*CTX-M is prevalent in both hospitals and community settings (Bauernfeind *et al.*, 1990; Cantón & Coque, 2006).

*bla*CTX-M results in a higher level of resistance towards cefotaxime, which is usually observed in all *bla* CTX-M producing isolates (Cantón & Coque, 2006). Carbapenems are stable against *bla*CTX-M-type enzymes; however, when they are hyperproduced and combined with decreased outer membrane permeability, *bla*CTX-M can result in resistance to carbapenems (D'Andrea *et al.*, 2013). The phenotypic cross-resistance of ESBL producing Enterobacteriaceae is associated with the existence of other genes, normally present in the same plasmid as the *bla*CTX-M gene. Most CTX-M producing organisms are resistant to fluoroquinolones (Cantón & Coque, 2006).

1.4.2.4 Epidemiology of ESBLs

ESBL-producing Enterobacteriaceae have been reported worldwide (Bevan *et al.*, 2017; Flokas *et al.*, 2017; Leopold *et al.*, 2014). According to the European Antibiotic Resistance Surveillance Network (EARS-NET) in 2013, 17 out of 22 countries in Europe recorded that 85 to 100% of *E. coli* isolates were ESBL-positive (EARS-Net, 2014). In Latin America in 2014, resistance to third-generation cephalosporins among *K. pneumoniae* ranged from 19%-87% (CDDEP., 2015). The International Network for Optimal Resistance Monitoring (INFORM) documented a 12% increase in ESBL-producing isolates in US hospitals from 2011 to 2013 (McDanel *et al.*, 2017). Fourteen percent of healthy individuals have been reported to be colonized with ESBL-positive isolates globally, with 2% prevalence rate in the USA (Karanika *et al.*, 2016). Thirty-five percent of *E. coli* isolates causing urinary tract infections (UTI) were classified phenotypically as ESBL-producers from 2009-2010 in Asia-Pacific regions (Hsueh *et al.*, 2011; Lu *et al.*, 2012).

The latest report from SMART (Study for Monitoring Antimicrobial Resistance Trends) revealed that ESBL-producing *E. coli* isolates from intra-abdominal infections in the Asia-Pacific region almost doubled between 2002-2010, to 40.8%. The rise of ESBL-producing bacteria has made the treatment of Enterobacteriaceae in the Asia-Pacific region very difficult (Lee *et al.*, 2013).
K. pneumoniae used to be recognized as the main carrier of ESBLs in nosocomial infections, but in recent years *E. coli* has become the major source of ESBLs, with a high rate in community settings (Cantón & Coque, 2006). The prevalence of community-acquired bloodstream infections due to ESBL-producing *K. pneumoniae* and *E. coli* have been reported as 55.5% and 16.5% respectively in China (Quan *et al.*, 2017).

In Africa, research on antibiotic resistance is localized in various countries but there is no research summary of the prevalence of resistance and the genes involved in Enterobacteriaceae (Storberg, 2014). Much of the data on antibiotic resistance in Enterobacteriaceae is from hospitals, and there is limited or no information on resistance in community-acquired infections (Ruppé *et al.*, 2009). In sub-Saharan Africa, resistance to the third-generation cephalosporins among Enterobacteriaceae in patients with community-acquired febrile illness was between 0-47% from 1990-2013 (Leopold et al., 2014). The ESBL prevalence in North Africa ranged from 12-99% in hospitals and 1-11% in communities (Khalaf *et al.*, 2009; Naas *et al.*, 2011). In East Africa, ESBL producing organisms were found in 38-63% of samples from hospitals and 6% of community samples (Beyene *et al.*, 2011; Kiiru *et al.*, 2012). The number of deaths due to pediatric sepsis caused by ESBL-producers is significantly higher than non-ESBL-producers; in tertiary hospitals in Tanzania children less than 8 years with sepsis due to an ESBL-producer had a mortality rate of 71% compared to 39% in those with non-ESBL producing organisms (Blomberg *et al.*, 2005; Lee *et al.*, 2013). In some parts of Central Africa, ESBL rates in hospital-acquired isolates were as high as 83%, with 17% in community samples (Lonchel *et al.*, 2012, 2013). ESBL resistance continues to increase in West Africa; at 40% in community and 63% in the hospitals (Feglo *et al.*, 2013; Tandé *et al.*, 2009) and 49.3% of Enterobacteriaceae isolates from Ghanaian hospital has also been identified as ESBL-producing (Obeng-Nkrumah *et al.*, 2015).

SENTRY antimicrobial surveillance in South Africa revealed that 36% of *K. pneumoniae* and 5% of *E. coli* were ESBL-positive from 1998 to 1999. Similarly, results of the Pan-European Antimicrobial Resistance using Local Surveillance (PEARLS) study from 2001 to 2002 in South Africa revealed that 34% of *K. pneumoniae* and 4.6% of *E. coli* were ESBL-producers (Bouchillon *et al.*, 2004). Sixty-two percent of *K. pneumoniae* isolates from seven government hospitals in South Africa were ESBL-producers in 2007 (Bamford *et al*., 2009). The prevalence of ESBL-producing Enterobacteriaceae in South Africa was 9-13% in hospital and 0.3-5% in communities while resistance to third-generation cephalosporins in *E. coli* and *K. pneumoniae* remained stable at 19% and 32% respectively from 2012 to 2014 (CDDEP, 2015). However, within the same period, Vasaikar and colleagues reported an increased prevalence of ESBLs in different provinces of South Africa, ranging from 36.1% to 68.3% (Vasaikar *et al.*, 2017).

*bla*SHV-2 has been reported in South Africa and Laos as the most prevalent *bla*SHV among *E. coli* isolates (Stoesser *et al.*, 2014; Storberg, 2014). However, a few *bla*SHV variants such *bla*SHV-30, *bla*SHV-23, *bla*SHV-12, and *bla*SHV-5 have also been documented (Liakopoulos *et al.*, 2016; Peirano *et al.*, 2011; Szabó *et al.*, 2005; Usha *et al.*, 2008). Three percent of ESBL producers in the fecal carriage of Cameroonian children in the community were *bla*SHV-12 (Lonchel *et al.*, 2012). *bla*TEM-1 is the most prevalent *bla*TEM among Enterobacteriaceae in South Africa in community and hospital settings (Storberg, 2014)

The first *bla*CTX-M gene was reported in South Africa in 2006 in a *K. pneumoniae* isolate (Elliott *et al.*, 2006). The *bla*CTX-M-15 gene has since also been reported in Durban and Pretoria (Usha *et al.*, 2008, Ehlers *et al.*, 2009). Ninety-five percent of ESBL-producing *E. coli* isolates from community hospitals in Cape Town expressed *bla*CTX-M genes, of which *bla*CTX-M-15 and *bla*CTX-M-14 were the most prevalent (Peirano *et al.*, 2011). A recent study conducted in Mthatha in the Eastern Cape of South Africa showed that 56.7% of clinical *Klebsiella* isolates harbor *bla*CTX-M (Vasaikar *et al.*, 2017). In Mali, Tunisia, and Cameroon the prevalence of *bla*CTX-M-15 among ESBL producers was 83%, 91%, and 96% respectively (Ouedraogo *et al.*, 2016). The global spread of *bla*CTX-M might be due to the combination of mobile genetics and specific clonal dissemination (Ouedraogo *et al.*, 2016).

The increasing prevalence of *bla*CTX-M in hospitals might be due to the introduction of these enzymes from the community rather than expansion in the hospitals as documented by previous studies (Overdevest *et al.*, 2011). Overdevest and colleagues found ESBL-producing Enterobacteriaceae in rectal swabs of 4% of patients before admission to hospital (Overdevest et al., 2011). Valverde and colleagues reported that the fecal carriage rate of ESBL producing Enterobacteriaceae in a Spanish community was 5.5% in hospitalized and 3.7% in healthy individuals in 2003 (Valverde *et al.*, 2004). Fecal carriage of *bla*CTX-M-producing Enterobacteriaceae has been observed in community settings in Asia, America and Europe (D'Andrea *et al.*, 2013). A community study in Phnom-Penh, Cambodia, revealed that all ESBL-producing isolates carried *bla*CTX-M (Ruppé *et al.*, 2009). There have also been cases of spread of *bla*CTX-M producers among household contacts (D'Andrea *et al.*, 2013). These findings are suggestive evidence that the community might be serving as a reservoir for *bla*CTX-M-producers (D'Andrea *et al.*, 2013) which may have originated in nosocomial settings and become established in the community. The danger of *bla*CTX-M carriage in the community is still unexplored (Ruppé *et al.*, 2009).

1.4.3 Carbapenemases

Carbapenemases have a wide hydrolytic spectrum and are capable of inactivating almost all β-lactams. There is a wide range of carbapenemases, and they are not all structurally related. The most dominant carbapenemase globally is *bla*KPC, however recently others such as *bla*NDM, *bla*IMP, *bla*VIM, *bla*GES, and *bla*OXA-48 have also been reported (Kocsis and Szabó, 2013). All the above-mentioned carbapenemases have been reported to occur on plasmids. In South Africa, the most prevalent carbapenemases are *bla*OXA-48 and *bla*NDM (Osei Sekyere, 2016). The main reservoir of Carbapenemase-Producing Enterobacteriaceae (CPE) is the intestinal tract, and fecal specimens are used to screen for CPE. It can be difficult to isolate CPE from stool samples because they form only a small proportion of the fecal microbial load (Yamamoto *et al.*, 2017).

1.4.3.1 Guiana-Extended-Spectrum Carbapenemase (blaGES)

*bla*GES-type enzymes are members of the Ambler class A plasmid-mediated β-lactamases. Although *bla*GES enzymes result in a phenotype similar to ESBLs, they have greater activity against carbapenems due to changes in their active site. The *bla*GES-type has 26 variants and they differ from each other by 1 to 4 amino acid substitutions (Frase *et al.*, 2009). *bla*GES-2, *bla*GES-4, *bla*GES-5, *bla*GES-11, *bla*GES-18, *bla*GES-20 have disseminated worldwide, with reports from Brazil, France and South Africa (Ribeiro *et al.*, 2014). Isolates producing *bla*GES enzymes have been isolated predominantly in Europe, South Africa and the Far East (Walther-Rasmussen & Høiby, 2007).

1.4.3.2 Klebsiella pneumoniae Carbapenemase (blaKPC)

*bla*KPC belongs to the molecular class A serine β-lactamases and functional group 2f (Wolter *et al.*, 2009). *bla*KPC-1-8 have been described (Lee & Burgess 2012) and South Africa was the first country on the African continent to report the presence of *bla*KPC (Brink *et al.*, 2012). *bla*KPC-producing organisms do not always show phenotypic resistance to carbapenems and may resemble ESBL producers (Wolter *et al.*, 2009). However, most hospital-acquired infections involving *bla*KPC are associated with therapeutic failure and a 50% mortality rate (Lee & Burgess 2012).

1.4.3.3 New Delhi Metallo- β-lactamase (blaNDM)

*bla*NDM is a class B Metallo- β-lactamase, which was first reported in 2008 and is the most recently recognized carbapenemase. *bla*NDM is predominantly found in *E. coli* and *K. pneumoniae* (Nordmann *et al.*, 2011). In the past years, 17 new variants of *bla*NDM have been reported with one or two amino acid substitutions. Studies to date only report plasmid-mediated *blaNDM* genes, especially in Enterobacteriaceae. *bla*NDM-positive isolates have been reported in over 40 countries across the globe. Cases of *bla*NDM-positive isolates have been reported on the African continent from a range of countries, including Algeria and Cameroon, with patients transferred from *bla*NDM endemic areas (Johnson & Woodford, 2013). However,

local dissemination of NDM has also been reported in South Africa (Brink *et al.*, 2012) and Kenya (Poirel *et al.*, 2011).

1.4.3.4 Imipinim-resistant Metallo-β-lactamase (blaIMP)

*bla*IMP is an Ambler subclass B1 metallo-β-lactamase that has developed activity against all β-lactams except aztreonam (Limbago *et al.*, 2011). The *bla*IMP-1 enzyme was first discovered in 1991 in Japan in *Serratia marcescens* and since then reports have been documented globally especially in Enterobacteriaceae (Liu, Pegg, *et al.*, 2012). Recently, the IMP metallo β-lactamase (MBL) has disseminated across the Enterobacteriaceae family, including *K. pneumoniae* and *E. coli* (Notake *et al.*, 2013).

1.4.3.5 Verona integron-encoded Metallo-β-lactamase (blaVIM)

*bla*VIM is a class B carbapenemase first detected in Italy in 1999 (Lauretti *et al.*, 1999). *bla*VIM-type enzymes can be categorized into 3 major classes according to amino acid sequences; *bla*VIM-1, -2 and -7. All other newly discovered *bla*VIM-types cluster with these three major variants, and differ by one or two amino acid substitutions. *bla*VIM-1 and *bla*VIM-2 are globally disseminated variants (Cornaglia *et al.*, 2011). In 2008, Algeria first reported *bla*VIM-19, which differs from *bla*VIM-1 and *bla*VIM-4 by one amino acid substitution, in Enterobacteriaceae (Robin *et al.*, 2010). In 2006 *bla*VIM-4 producing *K. pneumoniae* isolates were detected during a hospital outbreak in a Tunisia, demonstrating a possible spread in North Africa (Ktari *et al.*, 2006). In 2013, Morocco reported the first *bla*VIM-1 in *K. pneumoniae* isolates from a non-hospitalized patient (Barguigua, El otmani, *et al.*, 2013).

1.4.3.6 Oxacillinase β-lactamase (blaOXA-48)

*bla*OXA-48 is an Ambler class D β-lactamase. *bla*OXA-48 like variants have been described all over the world, with *bla*OXA-48 being the most predominant carbapenemase globally and the most commonly detected carbapenemase in Enterobacteriaceae. *bla*OXA-48 was first detected in

carbapenem-resistant *K. pneumoniae* from Istanbul, Turkey in 2001. *bla*OXA-48 is common in North and South Africa, Middle East, Turkey and India, which might serve as reservoirs since related clones from these countries have been observed in Europe and other parts of the world (Poirel *et al.*, 2012).

1.5.3.7 Epidemiology of Carbapenemases

Five European countries have reported increases in carbapenemase-producing Enterobacteriaceae (CPE) in 2013 (EARS-Net, 2014). In hospitals in the USA in 2013, rates of carbapenem resistance in *K. pneumoniae* and *E. coli* were 11 and 2% respectively. In India, 13% of *E. coli* isolates were resistant to carbapenems in 2013, and in 2014 57% of *K. pneumoniae* isolates were resistant to carbapenems (Storberg, 2014).

In South Africa, the first *bla*NDM carbapenemase was reported in 2010 and since then several cases have been recorded across the country. *bla*NDM-producing Enterobacteriaceae are becoming endemic, both localized and cases from the foreign countries have been reported (Jager *et al.*, 2015; Lowman *et al.*, 2011). Nineteen out of 70 CPE isolates received from public and private hospitals in South Africa were *bla*NDM-positive (Laxminarayan *et al.*, 2013). VIM-1 producing Enterobacteriaceae have been documented in South Africa (Jager *et al.*, 2015). In 2013, the prevalence of carbapenem resistance was 2 and 0.8% in *K. pneumoniae* and *E. coli* isolates respectively in South Africa (CDDEP., 2015). Although surveillance for CRE in South Africa is still based primarily on voluntary submission of isolates, a recent review confirmed approximately 2315 cases of CRE from 2000 to 2016, of which 49% were *Klebsiella pneumoniae* (Osei Sekyere, 2016). While carbapenem resistance is most likely to be associated with hospital acquired infections, there is little data from South Africa confirming what proportion of carbapenem resistant Enterobacteriaceae are hospital acquired.

1.5 Fluoroquinolone Resistance in Enterobacteriaceae

Quinolones are one of the world's most commonly prescribed antibiotics, and are used to treat bacterial infections such as UTI (Aldred *et al.*, 2014). Quinolones target two important enzymes in the bacterial cell; DNA gyrase (topoisomerase II) and DNA topoisomerase IV. These enzymes are involved in DNA replication and synthesis (Drlica *et al.*, 2009). Nalidixic acid was the first quinolone to be introduced in the 1960s (Aldred *et al.*, 2014). Subsequently, the fluoroquinolones (such as ciprofloxacin, levofloxacin, and moxifloxacin) were developed and introduced, with improved pharmacokinetics, as well as improved activity against Gram-positive organisms (Kocsis & Szabó, 2013).

Quinolone resistance can either be chromosomal or plasmid-mediated (Kocsis & Szabó, 2013). Mutations in the chromosomal genes encoding the gyrase and topoisomerase enzymes (*gyrA/B* and *parC/E*) are the commonest mechanisms of resistance. More recently described mechanism of resistance involve the plasmid-mediated *qnr*-family of genes (*qnrA, qnrB, qnrC, qnrD,* and *qnrS*). The first *qnr* gene was identified in *E. coli,* and it mediates resistance by protecting DNA gyrase from inhibition by fluoroquinolone antibiotics (Guan *et al.*, 2013).

Enterobacteriaceae can also develop resistance to quinolones through the acquisition of efflux pumps. The efflux pumps are made of a membrane fusion protein (AcrA), inner membrane pump (AcrB) and an outer membrane protein (ToiC). Hyperproduction of these proteins leads to fluoroquinolone resistance by actively exporting the antibiotic from the bacterial cell; as shown in Figure 1.3 (Kocsis & Szabó, 2013). Other plasmid-encoded genes which can play a role in quinolone resistance are *qepA* and *oqxAB* which also produce efflux pumps (Rodriguez-Martinez *et al.*, 2016). The enzyme aminoglycoside acetyltransferase *aac(6')-Ib-cr*, causes reduced susceptibility to aminoglycosides and to some fluoroquinolones.

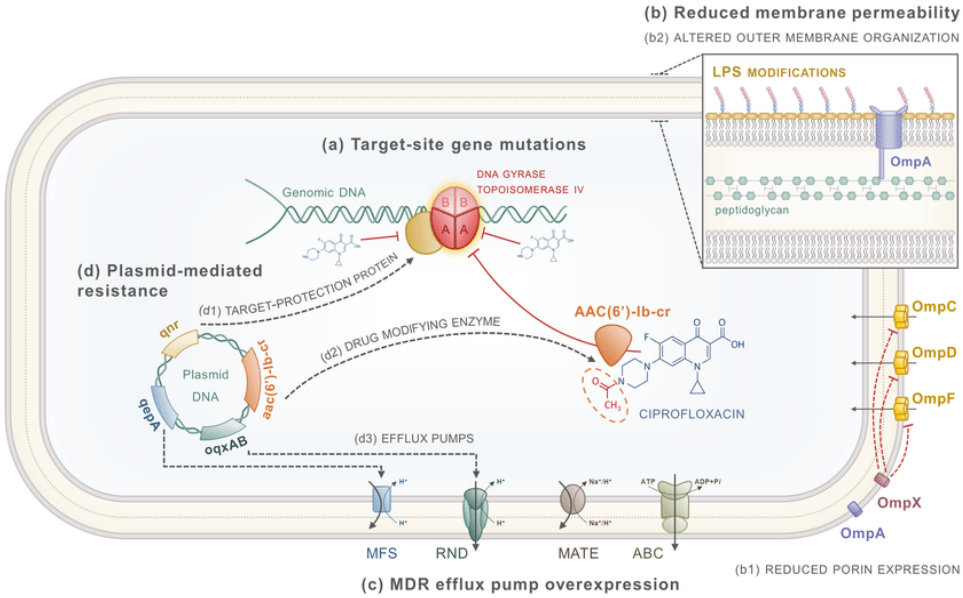


Figure 1.3: Fluoroquinolone resistance mechanisms. (a). Chromosomal mutations in the QRDRs genes (*gyrA* and *parC*) alter the target site, reducing the binding affinity of the antibiotic. (b). Reduced porin expression due to chromosomal mutations (c) QepA and OqxAB efflux pumps play a role in quinolone resistance (d) Plasmid-mediated quinolone resistance genes (*qnrA, qnrB,* and *qnrS*) promote the resistance by protecting DNA gyrase and topoisomerase IV from quinolone inhibition. **(Adapted from Correia et al. 2017)**

1.5.1 Plasmid-mediated Quinolone Resistance

It is important to note that plasmid-mediated *qnr* genes by themselves do not confer resistance, but promote the selection of microorganisms with higher Minimum Inhibition Concentrations (MIC), therefore allowing the acquisition of other resistance mutations (Guan *et al.*, 2013). A recent study in Morocco showed that some isolates expressing Plasmid-mediated Quinolone Resistance (PMQR) were not resistant to fluoroquinolones while others which have reduced susceptibility to fluoroquinolones do not express PMQR (Benaicha *et al.*, 2017).

PMQR genes, such as *qepA* and *qnr*, are mostly co-expressed with ESBLs because they are usually located on the same genetic elements (Crémet *et al.*, 2011). Fluoroquinolone-resistance genes such as *qnrA* and *qnrB* have been co-expressed with CTX-M genes. *qnrA* has been

detected in *blaCTX-M*-9 and *blaCTX-M*-14 producing isolates; whereas *qnrB* and *aac(60)-Ib-cr* have been linked with *blaCTX-M*-15 (Cantón & Coque, 2006).

1.5.1.1 qnrA

qnrA was the first plasmid-mediated quinolone resistance gene, described in *K. pneumoniae*, in the USA in 1988. Since then several plasmid-encoded quinolone resistance genes have been described including *qnrB* and *qnrS* (Qian *et al.*, 2017). The *qnrA* gene is said to have originated from *Shewanella algae* (Guan *et al.*, 2013), and has also been identified in phages from water samples (Rodriguez-Martinez *et al.*, 2016).

When *qnrA* is co-expressed with *acc-(6')-Ib-cr* the level of ciprofloxacin resistance is increased 4-fold compared to *qnrA* alone (Vali *et al.*, 2015). *qnrA* confers resistance to quinolones such as nalidixic acid and increases the MIC 20-fold. Conjugation with a *qnrA* plasmid using *E. coli* J53 increased resistance to nalidixic acid between 12.5 and 250-fold (Guan *et al.*, 2013).

1.5.1.2 qnrB

qnrB was first reported in 1998 and has diversified over the years resulting in many variants (Hong *et al.*, 2009). Among all the *qnrB* variants, *qnrB31* and q*nrB32* have the highest amino acid similarity. Of the *qnr* genes involved in quinolone resistance, *qnrB* is most prevalent. *qnrB* confers low-level resistance to quinolones, but also has a unique characteristic which enables it to mimic DNA and serve as a substrate for DNA gyrase (Wang *et al.*, 2011).

qnrB has been found to be widely disseminated in South America (Armas-Freire *et al.*, 2015). A study in Ecuador by Armas-Freire suggested that *qnrB* is predominant in rural settings where antibiotic use is low compared to urban nosocomial settings (Armas-Freire *et al.*, 2015). Some other studies have demonstrated that *qnrB* is predominant in commensal *E. coli* that were isolated from healthy children in Peru and Bolivia (Pallecchi *et al.*, 2010). A recent study in American Crows showed that 25% of their fecal samples contained *qnrB* (Halová *et al.*, 2014).

Reports suggest that there might be a link between plasmid-mediated quinolone resistance and extended spectrum β-lactamases, with *bla*CTX-M-15 and *bla*SHV-ESBLs located on the same plasmid as *qnrB* (Strahilevitz *et al.*, 2009)

1.5.1.3 qnrS

qnrS was first reported in *Shigella flexneri* (Poirel *et al.*, 2006) and has 9 known variants (Dobiasova *et al.*, 2015). Two variants of *qnrS*, i.e. *qnrS1* and *qnrS2*, have been identified which have 40% and 59% similarity to *qnrA1*, respectively (Cattoir *et al.*, 2007).

qnrS is the most frequently isolated plasmid-mediated quinolone resistance gene in *K. pneumoniae* and *E. coli* in China (Pasom *et al.*, 2013). Four percent of clinical *E. coli* isolates harbored *qnrS* in China (Jiang *et al.*, 2014). *qnrS1* is one of the most common plasmid-mediated quinolone resistance mechanisms recently reported in Durban, South Africa (Osei Sekyere & Amoako, 2017).

qnrS has been isolated from a number of environmental and agricultural settings, including wastewater, aquatic birds and farm animals (Colomer-Lluch *et al.*, 2014; Dobiasova *et al.*, 2015). There have been several reports of *qnrS*-positive isolates co-expressing ESBLs (Strahilevitz *et al.*, 2009), and other antibiotic-resistance genes have also been located on the same plasmid as *qnrS* (Dobiasova *et al.*, 2015). *bla*CTX-M has been detected in *qnrS*-positive *E. coli* isolates. Among 18 *qnrS*-positive isolates from pigs in Taiwan, 12 had a confirmed *bla*CTX-M-1 and three had *bla*CTX-M-15 (Kuo *et al.*, 2009). This new information adds to the fact that there might be a close link between *qnrS* and *bla*CTX-M in *E. coli* isolates, selecting for diverse resistance mechanisms in *E. coli* isolates in farm animals. A clinical *E. coli* isolate from China carried *bla*OXA-181 and *qnrS1* on the same plasmid (Pulss *et al.*, 2017) and *aac(6')-lb-cr* has also been linked with *qnrS* (Ory *et al.*, 2016).

1.5.2 Chromosomal Quinolone Resistance Mutations

The resistance of *E. coli* isolates to quinolones is often linked to alterations in DNA topoisomerase II. Topoisomerase II has two subunits i.e. *gyrA* and *gyrB* (Mehla & Ramana, 2016). Topoisomerase IV consists of two subunits (*parC and parE*) which are homologous to *gyrA* and *gyrB*, respectively. A recent study has shown that quinolones interact with topoisomerase IV through a water-metal ion bridge, and partial disruption of this bridge, resulting from mutation, leads to decreased susceptibility (Aldred *et al.*, 2014).
GyrA has a distinct characteristic which induces a negative supercoil into DNA, which helps in the replication of the DNA (Mehla & Ramana, 2016). GyrA has a tyrosine at its active site which is responsible for DNA cleavage and ligation. GyrB has ATPase activity and is necessary for all catalytic activities (Aldred *et al.*, 2014).

Chromosomal mutations in *gyrA* play a significant role in quinolone resistance (Gu *et al.*, 2017). Mutations are more often seen in *gyrA* as compared to *parC;* and most reported alterations are at the serine codon 83, as shown in Table 1.1 (Onseedaeng & Ratthawongjirakul, 2016). This mutation is the initial step in acquiring fluoroquinolone resistance and most often results in high-level nalidixic acid resistance (Abdi-Hachesoo *et al.*, 2013). Asp87 and Ser83 substitutions are the most frequently reported in *E. coli* (Piekarska *et al.*, 2015). The number of DNA alterations in quinolone resistance determination regions of *gyrA* is significantly linked to MICs of quinolones (Liu, Liao, *et al.*, 2012). It has been hypothesized that *gyrA* appears as a primary fluoroquinolone target in Enterobacteriaceae (Piekarska *et al.*, 2015). Mutations in *parC* are the next step leading to high level fluoroquinolone resistance (Liu, Liao, *et al.*, 2012). Topoisomerase IV (ParC) is the secondary target of quinolones if mutations in gyrase are present (Chenia *et al.*, 2006). Ser80 and Glu84 substitutions in ParC are the most frequently reported mutations in *E. coli* isolates with reduced susceptibility to fluoroquinolones (Piekarska *et al.*, 2015). Mutations in the quinolone resistance determination region of *parC* result in high level fluoroquinolone resistance (Gu *et al.*, 2017). Many isolates that showed resistance to levofloxacin and ciprofloxacin had alterations in ParC i.e. Ser87Leu and Ala88Pro mutations (Nouri *et al.*, 2016). A *K. pneumoniae* isolate with mutations in the *parC* gene coupled with *aac(6')-Ib-cr* and *qnrB1* was shown to have a ciprofloxacin MIC of 16 mg/L. Mutations in *parC*

alone cause higher ciprofloxacin MICs, ranging from 0.125 to 128 mg/l (Liu, Liao, *et al.*, 2012). Isolates that have mutations in both *gyrA* and *parC* genes have high-level resistance to ciprofloxacin and levofloxacin, compared to those with mutations in *gyrA* alone (Nouri *et al.*, 2016).

Table 1.1: Frequently reported fluoroquinolone resistance mutations in *GyrA* and *ParC*

GyrA		***ParC***	
Mutations	References	Mutations	References
S83L	(Gu et al. 2017)	S80I	(Liu *et al.*, 2012)
S83A	(Liu *et al.*, 2012)	S80R	(Liu *et al.*, 2012)
S83I	(Fu *et al.*, 2013)	E84K	(Liu *et al.*, 2012)
S83F	(Fu *et al.*, 2013)	E84G	(Liu *et al.*, 2012)
S83Y	(Fu *et al.*, 2013)	E84V	(Onseedaeng&Ratthawongjirakul 2016)
T83I	(Nouri et al. 2016)	Met86Tp	(Gu et al. 2017)
Al84V	(Piekarska et al. 2015)	S87L	(Nouri et al. 2016)
D87N/G	(Gu et al. 2017)	A88P	(Nouri et al. 2016)
D87E	(Piekarska et al. 2015)	A90V	(Onseedaeng&Ratthawongjirakul 2016)
D87Y	(Onseedaeng & Ratthawongjirakul 2016)	A108T	(Onseedaeng&Ratthawongjirakul 2016)
D87A	(Liu *et al.*, 2012)	Al108V	(Liu *et al.*, 2012)
D87H	(Liu *et al.*, 2012)	S129P	(Gu et al. 2017)
I89F	(Liu *et al.*, 2012)	S129A	(Norouzi *et al.*, 2014)
K154R	(Norouzi *et al.*, 2014)	A141V	(Norouzi *et al.*, 2014)
S171A	(Norouzi *et al.*, 2014)		
V190G	(Norouzi *et al.*, 2014)		
H211Y	(Gu et al. 2017)		

1.5.3 Epidemiology of Quinolone Resistance

From 1997 to 1999 approximately 50% of community and 60% of hospital-acquired *E. coli* isolates in China were resistant to ciprofloxacin (Guan *et al.*, 2013). In Thailand, a study aimed at investigating the prevalence of antibiotic resistance in fecal flora of patients undergoing a transrectal ultrasound-guided needle biopsy of the prostate (TRUSB) showed that the prevalence of fluoroquinolone and cephalosporin-resistant *E. coli* and *K. pneumoniae* was high in patients who had fluoroquinolone prophylaxis (Siriboon *et al.*, 2012). A study in Paris showed the emergence of quinolone resistance in *E. coli* isolates in 10 of 40 healthy adults exposed to 14 days of oral ciprofloxacin, although resistance was absent at baseline (Fantin *et al.*, 2009).
Interestingly, rooks may be a reservoir for the spread of quinolone-resistant bacteria in Europe. A recent study in Europe showed the prevalence of ciprofloxacin-resistant Enterobacteriaceae to range from 3% to 92% in rooks (Halová *et al.*, 2014). Gurnee *et al* reported that 20% of children and their mothers were colonized by ciprofloxacin-resistant Gram negative bacteria in the USA from January, 2010 to May, 2013 (Gurnee *et al.*, 2015).

In Africa, ciprofloxacin-resistant *Klebsiella* isolates from outpatient departments range from 0-47% (Tansarli *et al.*, 2013). In South Africa ciprofloxacin-resistant Enterobacteriaceae ranged from 0-15% in public hospitals in 2007, while in 2010, 18% were ciprofloxacin-resistant (Bamford *et al.*, 2009, 2011). Sixty percent of *Klebsiella* isolates from the NHLS laboratory in the Eastern Cape of South Africa are ciprofloxacin-resistant (Vasaikar *et al.*, 2017), whereas it is not clear what proportion were from community acquired infections. Although limited, these data do suggest that FQ resistance is relatively common, and thus of clinical concern.

1.6 The Effect of Antibiotic Exposure on Selection of Resistance

There is good evidence that increased consumption of antibiotics drives antibiotic resistance (Cantón & Morosini, 2011). Yet it is poorly defined as to whether the same can be expected *in vitro*. Few *in vitro* studies and animal models have provided knowledge of how resistance emerges (Craig, 1998; Sykes, 2010). Previously it was believed that resistance could only emerge as a result of exposure to a lethal antibiotic concentration [i.e. concentrations above the

organism's minimum inhibition concentration (MIC) for the particular antibiotic]. The concern that sublethal concentrations (below MIC) could select for resistance was nearly ignored (Andersson & Hughes, 2014). However, recent studies begin to reveal that low-level antibiotic concentration is a major selector of resistant mutants (Andersson & Hughes, 2014).

Exposure of *E. coli* to ampicillin (1 µg/ml), norfloxacin (15 µg/ml) and kanamycin (3 µg/ml) resulted in a significant increase in the proportion of resistant isolates compared to isolates not exposed to the antibiotic. The MIC of the isolates selected after ampicillin exposure was 5-fold higher than wild-type, while the MICs of kanamycin and norfloxacin were approximately 2 and 3-fold higher, respectively. Ampicillin exposed isolates were resistant to ampicillin and norfloxacin while resistant isolates after norfloxacin and kanamycin exposure were resistant to ampicillin (Kohanski *et al.*, 2010). Resistant *E. coli* selected from media containing a low concentration of tetracycline and chloramphenicol were 6-8 fold less susceptible to fluoroquinolones (norfloxacin, ofloxacin, ciprofloxacin, and nalidixic acid) than the wild-type (Cohen *et al.*, 1989). In another experiment, *E. coli* and *Salmonella typhimurium* were exposed to ciprofloxacin and streptomycin at 0.1x MIC of their susceptible wild-type; this exposure led to increased resistance. After several generations, *E. coli* had a 2-fold higher MIC than that of the wild-type strain after ciprofloxacin exposure. The MIC of *S. typhimurium* was 8-fold higher than the wild-type strain after being exposed to streptomycin (Gullberg *et al.*, 2011). Resistant *E. coli* isolates selected by exposure to sub-MIC concentrations of oxacillin were also resistant to ciprofloxacin and pre-treatment with sub-MIC levels of vancomycin led to gentamicin resistance (Johnson & Levin, 2013). These data show that sublethal antibiotic concentrations do not only enrich for existing mutants but may also select for *de novo* resistance in wild-type susceptible populations (Gullberg *et al.*, 2011). Exposure to low concentrations of antibiotics can select for low-level resistance which can gradually lead to high-level resistance (Baquero, 2001). Antibiotic selection enriches the number of resistance genes in a microbial population, but these genes might exist before the selection (Cantón & Morosini, 2011).

Antibiotic absorption to different parts of the body is irregular and mostly depends on the pharmacodynamics and pharmacokinetics of the antibiotics (Baquero & Negri, 1997). The concentration of antibiotic at these sites may be sublethal and is probably more likely to select

for resistance; 20-80% of antibiotics are recovered in stool and most of the concentrations of antibiotics in stool are sublethal and can select for resistance (Baquero & Negri, 1997; Johnson, 2006).

It is now clear that selection of resistance to antibiotics can occur not only at high concentrations but at a wide range of concentrations. However, it is difficult to define the lowest concentration of antibiotics that can still select for resistance. The few studies done on *in vitro* antibiotic exposure have been on selected antibiotics. It is important to investigate this in a wider class of antibiotics. The medical significance of acquisition of resistance caused by sub MIC antibiotic exposure is under-explored (Andersson & Hughes, 2014).

1.7 Surveillance as a Strategy to Combat Antibiotic Resistance

Surveillance is one of the key strategies to combat AMR. In May 2015, the WHO adopted a global plan which focuses on ensuring that antibiotics are of good quality, effective and available to those who need them. It is anticipated that individual countries will develop their own national plans on antimicrobial resistance in line with WHO global plan (WHO, 2016). Few member states have regulations on antimicrobials (CDC, 2013).
The following actions can help monitor and control antimicrobial resistance globally: surveillance of antimicrobial resistance and tracking of resistance patterns by DNA sequencing (CDC, 2013).

Antibiotic resistance surveillance is needed for several reasons: it enables potential problem areas to be identified early and tackled; it is important for any antimicrobial stewardship initiative to accomplish its purpose; it is necessary for monitoring national and regional antibiotic consumption and to monitor the antibiotic prescription rate in private and public health facilities (Suleman & Meyer, 2012).

Many countries have surveillance systems in place to report and track antibiotic resistance in community and hospital-acquired infections, including UK, USA, China, India, Vietnam, New Zealand, and South Africa. South Africa presently compiles data from the government and

private sectors using laboratory-based surveillance for the ESKAPE organisms (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp.). In the government sector, the laboratory data are compiled by the National Institute for Communicable Diseases (NICD) and includes data gathered from public hospitals by the GERMS-SA network (Group for Enteric, Respiratory and Meningeal disease surveillance in South Africa), a nationwide clinical microbiology network. Data from the private sector are collected by the South African Society of Clinical Microbiology. The South African Society of Clinical Microbiology (SASCM) and the South African Antibiotic Stewardship (SAASP) Partnership are compiling these sets of data (CDDEP, 2015).

The 2014 antibiotic resistance surveillance report released by WHO revealed a high rate of resistance in pathogens that cause community and nosocomial infections and has also pinpointed the absence of population-based AMR surveillance that could provide good information on the dissemination, mortality and economic impact on society (WHO, 2014).

It is very difficult to understand the mode of transmission of antibiotic resistance because most studies focus on tertiary hospitals and those who consider community enrolment, recruit only a small portion of the entire population (Grundmann, 2014).

Information in the above-mentioned areas on surveillance is needed to draft guidelines for public health intervention (FAO *et al.*, 2017). The ability to identify and profile novel antibiotic resistance mechanisms is necessary to ensure effective surveillance (WHO, 2015). However, detection of antibiotic mechanisms requires specialized skills and equipment; for this reason, global antibiotic surveillance cannot be built instantly but requires a guided developmental process (Grundmann, 2014). The world currently needs surveillance that produces quality data; this is vital for comprehensive global strategies and public health actions to control antibiotic resistance (WHO, 2014).

Exposure to antibiotics plays a crucial role in selecting for resistant bacteria. This selective pressure is due to the abuse and inappropriate use of antibiotics in patients, farm animals and in the environment. The environment has also contributed largely to the spread and transmission of

resistance. Major environmental sources include manure from farm animals, wastewater plants and hospital sewage. There is a need for policy-makers to devise strategies to minimize the risk of environmental exposure. The use of antibiotics for prophylactic treatment in veterinary medicine has caused the spread of resistance through the food chain. Interventions to tackle antibiotic resistance in animal farming could include improved hygiene in animal houses and educating veterinarians and other animal husbandry practitioners. The European Food Authority is playing a significant role in identifying risks of antibiotic resistance associated with the food industry (Roca *et al.*, 2015).

1.7.1 Limitations to antibiotic resistance surveillance in Africa

Antibiotic resistance is not only a clinical issue but also a global threat; the WHO and CDC have called on all stakeholders to join the fight against antibiotic resistance (Roca *et al.*, 2015). WHO recognizes laboratory-based surveillance as a priority for the global containment of antibiotic resistance; but for this to be a reality in Africa, state of the art laboratories in various health sectors of the continent will be needed, this will also help policymakers to make decisions concerning antibiotic resistance (WHO 2001).

A recent study by WHO and the NICD in South Africa revealed weaknesses in the laboratory capacity of most African countries and their inability to do valid susceptibility testing. There is a lack of proper framework which collaborates and identifies novel antibiotic resistance mechanisms and resistance profiles in Africa. This is a limitation to the progress of continental surveillance. Continental antibiotic resistance surveillance will help us know the impact of interventions in Africa because policymakers are now able to update the antimicrobial resistance policy (Ndihokubwayo *et al.*, 2013).

1.8 Problem Statement

There is a lack of information related to carriage of antibiotic resistance and mechanisms of resistance in children, and in the community in general; and the impact of antibiotic exposure on the development and carriage of resistance is poorly understood.

1.9 Study Design

The Tuberculosis Child Multidrug-resistant Preventive Therapy Trial (TB-CHAMP) is a phase III cluster randomized placebo-controlled trial to evaluate the efficacy of levofloxacin for multidrug-resistant (MDR) tuberculosis (TB) prophylaxis in children (<5 years) whose contacts have confirmed MDR-TB. This study provides an opportunity to investigate the carriage of antibiotic resistance and mechanisms of resistance in children in the community, and to investigate the impact of antibiotic exposure on resistance rates. Baseline stool samples were collected from children enrolled in TB-CHAMP for surveillance of resistance.

1.10 Aim

The aims of this study are to describe baseline rates of antibiotic resistance carriage in Gram-negative organisms in children in the community and to investigate the effects of antibiotic exposure on the transfer and acquisition of resistance in stool using an *in vitro* model.

Chapter 2 : Culture-based Screening for Resistant *E. coli* and *Klebsiella spp*. in Stool Samples

2.1 Introduction

Most of the current data and research dealing with antimicrobial resistance has been conducted on adults. Data on pediatric antimicrobial resistance is lacking, and where data is available, it focuses on hospitalized patients (Bryce *et al.*, 2016). There is therefore minimal data on antimicrobial resistance in children in a community setting. This is true for South Africa and other countries in Africa and it is of much interest. Community-acquired infections are a common cause of morbidity and mortality among children, and inappropriate antimicrobial choices are associated with worse clinical outcomes.

Efforts to describe rates of antimicrobial resistance in the community have been limited by aggregated laboratory data with little or no differentiation between community and hospitalized patient, as well as sometimes small sample sizes (Ashley *et al.*, 2011). Various culture-based screening methods have been described to identify ESBL producing organisms, using ceftazidime or cefpodoxime disks on McConkey agar and CHROMagar ESBL (Gazin *et al.*, 2012; Huang *et al.*, 2010). All these methods have yielded good results, however in this study we compared the use of cefpodoxime disks on McConkey agar to CHROMagar ESBL on a subset of isolates in order to identify the most effective, readily available and cheap method. The methods employed in this chapter were intended to maximize the chance of isolating ESBL producing *E. coli* and *Klebsiella* spp isolates, and to provide a representative collection of non-ESBL producing isolates both to screen for isolated fluoroquinolone and carbapenem resistance and to establish a bank of community-associated susceptible wild-type isolates.

The aim of this chapter is to describe the antibiotic susceptibility profiles of *E. coli* and *Klebsiella* isolates obtained from stool samples from healthy children in Cape Town. Subsequent chapters will describe additional molecular analyses of resistance mechanisms.

2.2 Materials and Methods

2.2.1 Study Population

This study forms part of a parallel-group, two-arm, cluster-randomized, double-blind placebo-controlled tuberculosis (TB) prevention trial (TB-CHAMP), which focuses on evaluating the efficacy of levofloxacin prophylaxis for MDR-TB (http://www.isrctn.com/ISRCTN92634082). Children less than 5 years whose parents or household contacts have confirmed MDR-TB are randomized to either 6 months of levofloxacin or 6 months of placebo, in a 1:1 ratio.

The TB-CHAMP project is being conducted at the Desmond Tutu TB Centre (DTTC), Stellenbosch University. The Center has two clinics in Cape Town, South Africa (DTTC Tambo Road Clinic and DTTC Beautiful Gate Clinic) where the participants are being enrolled. Tambo Road is in Khayelitsha, an informal settlement mostly populated by black Africans. Beautiful Gate, Philippi is a farming area which is mostly dominated by "peasant farmers". While participants are drawn from across Cape Town, the majority live in poor, high-density, urban areas.

2.2.1.1 Participant Inclusion Criteria

1. Child <5 years who is a household contact of an enrolled adult MDR-TB index case diagnosed during the previous 6 months
2. Primary residence in the household of the adult MDR-TB index case
3. Consent from the parent or legal guardian for the child for HIV testing (both HIV-infected and uninfected children will be included)
4. Consent obtained from the parent or legal guardian for the child to be enrolled

2.2.1.2 Child Participant Exclusion Criteria

1. TB disease at enrolment
2. Currently on isoniazid (INH) or a fluoroquinolone (FQN) (e.g. levofloxacin, moxifloxacin ofloxacin or ciprofloxacin) for ≥14 days

3. Treated for TB in the previous 12 months
4. Known concurrent exposure to an INH-susceptible (including rifampicin-monoresistant) index case
5. Children with myasthenia gravis or Guillain-Barré syndrome

2.2.2 Ethical Consideration

This study is part of a large research study (TB-CHAMP) for which ethics was approved by the Health Research Ethics Committee (HREC) at Stellenbosch University (S17/11/269). Provision for the collection of stool samples from participants is included in the TB-CHAMP study protocol and the informed consent process for TB-CHAMP. Informed consent was obtained from the parent or legal guardian of the participants. All samples and isolates were identified by study numbers, and no patient identifiers were captured. All data were maintained on a password-protected database, and all paper records were locked away.

2.2.3 Sample Collection

Prior to the initiation of prophylactic treatment or placebo, baseline samples were collected from enrolled children. Stool samples were collected by nurses/counselors per DTTC stool collection SOP (Appendix 1), making sure that urine or other environmental samples did not contaminate the container. Six scoops of formed stool or 3 scoops of loose stool were collected using a 2.5 ml spoon (Lasec, South Africa) and mixed several times for a uniform mixture (only urine-free stools were mixed). The stool containers were sealed, and the outside decontaminated with 70% ethanol. The containers were placed in a sealable plastic bag and labeled with a Subject Event barcode. The nurses/counselors then complete the requisition form (Appendix 2). The samples were stored in a fridge at the clinic (2-8°C) until transported to the laboratory.

In cases where stool could not be collected at the clinic by nurses/counselors, verbal instruction regarding the method of collection was given to the caregivers by the nurses/counselors together with an instruction leaflet (Appendix 3) and the stool collection kit without a requisition form.

The stool collection kit included a plastic leakproof screw-cap specimen container with a 2.5 ml spoon, leakproof bag, an opaque carrier, labels, a polystyrene cool box, and ice bricks.

The caregivers were encouraged to put the stool sample in the collection container as previously described and note the time and date of collection (either the night before or on the morning of the clinic visit). The caregivers were also entreated to put the sample on ice bricks in a cool box or in the coolest place in the house, until delivered to the clinic or picked up by the driver.

The sample was brought to Division of Medical Microbiology in a cooler box with an ice pack (2-8°C) during transport. Upon arrival at the medical microbiology laboratory, a swab of the stool was transferred to Cary Blair medium (Diagnostic Media Products, NHLS, South Africa) and stored at 4°C. The remaining sample was frozen at -80°C. The maximum time for which the samples were stored in Clary Blair before inoculation onto culture media was 7 days.

2.2.4 Bacterial Culture

Samples were inoculated onto CHROMagar ESBL (Biomerieux, France) for screening for ESBL-producing organisms. These agar plates have a reported sensitivity for detection of ESBLs of >95% (Gazin *et al.*, 2012); the medium contains a broad-spectrum β-lactam which is intended to select for putative 3rd generation cephalosporin-resistant Enterobacteriaceae. The chromogens in the medium allow for the differentiation of various species in the family Enterobacteriaceae by producing colored colonies specific to each species. Isolates of *E. coli* and *Klebsiella* spp. from the chromogenic medium were selected for further analysis as detailed below.

Samples were also inoculated onto standard McConkey agar (NHLS Media Laboratory, Greenpoint, South Africa) with the addition of a 10 µg cefpodoxime and a 10 µg ertapenem disc (Mast, Laboratories, UK). Cefpodoxime is a 3rd generation cephalosporin and ertapenem is a carbapenem; isolates within the zone of inhibition were followed up as putative ESBL and/or carbapenemase producers respectively. Colonies were presumptively identified as either *E. coli* or *Klebsiella* spp. based on fermentation of lactose (pink-pigmented colonies) as well as the typical colony morphology. Suspected *E. coli* and *Klebsiella* spp. isolates growing outside of the

zone of inhibition were followed up in an attempt to obtain susceptible isolates. The use of the cefpodoxime disc enabled the comparison between the "low cost" screening using discs with the more expensive CHROMagar plates.

All 69 isolates (whether from chromogenic medium or McConkey agar) were re-inoculated onto tryptose blood agar plates (TBA) (NHLS Media Laboratory) and incubated under aerobic conditions at 37˚C overnight. Single isolates from the TBA plate were stored in Microbank beads (Pro Lab Diagnostics, Canada) at -80°C for further analysis.

2.2.5 Identification and Antibiotic Susceptibility Testing

All isolates were identified using API10S kits or API20E kits (BioMerieux, France), following the manufacturer's instructions and based on >90% identity. Standard Kirby Bauer disc diffusion testing (Bauer *et al.*, 1966) was performed to determine susceptibility to penicillins, fluoroquinolones, aminoglycosides, cephalosporins, and carbapenems. Kirby-Bauer disc diffusion uses antibiotics impregnated into a filter-paper disc; these discs are placed onto an agar plate which has been inoculated with a standard inoculum of the organism in question. The antibiotics diffuse into the agar, resulting in inhibition of bacterial growth around the disc. The diameter of the zone is measured and interpreted using established interpretive criteria to classify isolates as susceptible, intermediate or resistant.

Bacterial isolates were retrieved from Microbank beads and inoculated onto fresh TBA plates. A single colony was streaked onto TBA plates and incubated overnight at 37°C under aerobic condition to obtain pure cultures. Three to four colonies were inoculated in 3 ml of purified water in small glass tubes using sterile swabs and vortexed briefly. The turbidity of the suspension was determined using the DensiCHEK (Biomérieux, France) to obtain 0.45-0.5 McFarland (equivalent to 1 - 2 x 10^8 CFU/mL). Clearly labeled Mueller Hinton agar plates (NHLS Media Laboratory) were inoculated using fresh swabs, and the plate was rotated to ensure that the entire surface of the plate was covered. The selected antibiotics were placed onto the agar plate using an antibiotic disc dispenser (Mast Laboratories, UK). The plates were incubated aerobically for 16 - 18 hours at 37°C. Every procedure was carried out on a

decontaminated bench and around a Bunsen burner to reduce the risk of contamination. After incubation, the zone diameter of each antibiotic was measured and interpreted using the Clinical and Laboratory Standards Institute (CLSI) 2016 guidelines. Multi-drug resistant (MDR) isolates were defined as being resistant to one agent in at least three different classes of antibiotics (Basak *et al.*, 2016)

Suspected carbapenem non-susceptible isolates (based on disc diffusion results) were confirmed using gradient diffusion strips (Liofilchem s.r.l., Italy) for determination of ertapenem, imipenem, and meropenem MICs. The strips are impregnated with the antibiotic in a gradient. One end of the strip has a high concentration, decreasing over the length of the strip. The strips are placed on a previously inoculated agar plate and release the antibiotic into the agar. After incubation, the growth around the strip can be seen as a symmetrically inhibited ellipse. Mueller Hinton agar plates were inoculated as described above, and ertapenem, imipenem and meropenem strips were placed at the middle of the agar plates and incubated as previously described. The plates were read and interpreted using CLSI 2016 guidelines.

2.3 Results

2.3.1 Isolate Numbers

Stool samples were collected from a total of 50 participants of the TB-CHAMP study at baseline. Fifty-two *E. coli* isolates were obtained from 43 (86%) of the participants, and seventeen *Klebsiella* spp (*K. oxytoca* and *K. pneumoniae*) were isolated from 14 (28%) of the participants (Figures 2.1 & 2.2). Seven of the participants were colonized by neither *E. coli* nor *Klebsiella* spp.

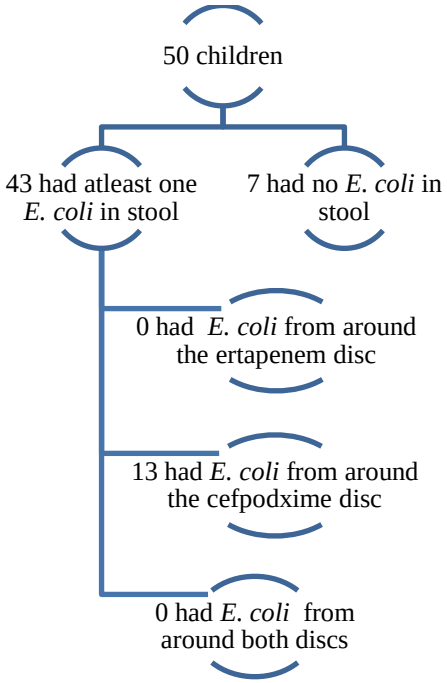


Figure 2.1: The proportion of children with *E. coli* isolates resistant to the cefpodoxime and ertapenem antibiotics.

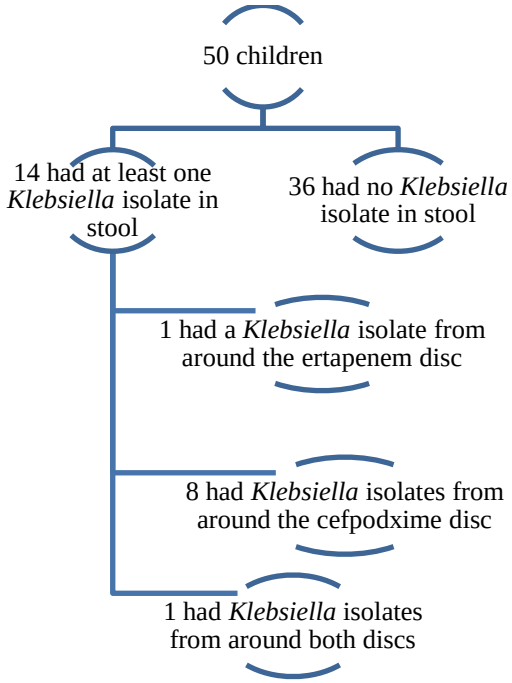


Figure 2.2: The proportion of children with *Klebsiella* isolates resistant to the cefpodoxime and ertapenem antibiotics

All samples where isolates were visible in the zone of inhibition around cefpodoxime on the McConkey agar also had isolates which were identified as putative ESBL producers on the CHROMagar ESBL. The yield from both approaches was evaluated for the first 30 samples and since the performance of cefpodoxime on McConkey agar and CHROMagar ESBL was identical (100%), the former was adopted for the rest of the study.

2.3.2 AST

2.3.2.1 Disc Diffusion

Of the 52 *E. coli* isolates, 33 (63%) were ampicillin-resistant, 4 (8%) β-lactamase inhibitor-resistant, 21 (40%) quinolone-resistant, 3 (6%) aminoglycoside-resistant and 13 (25%) cephalosporin-resistant. All 17 of the *Klebsiella* isolates (100%) were ampicillin-resistant, 4 (24%) β-lactamase inhibitor-resistant, 6 (35%) quinolone-resistant, 6 (35%) aminoglycoside-resistant, 8 (47%) cephalosporin-resistant and 1 (6%) carbapenem-resistant. However, as these proportions are influenced by the sampling strategy, and by the fact that multiple isolates were

obtained from some participants, it is more useful to present the data as a percentage of the children who carried isolates that were non-susceptible to the various antimicrobials, as shown in Table 2.1. Based on the disc diffusion susceptibility testing, 21 (42%) of the participants were colonized by quinolone-resistant isolates and 18 (36%) were colonized by cephalosporin-resistant isolates (possible ESBL-producers). All participants from whom cephalosporin-resistant isolates were obtained also carried cephalosporin-susceptible isolates.

Overall, sixty-six percent (66%) of the participants were colonized by isolates resistant to at least one antibiotic, while 17 (34%) of the participants were colonized by MDR isolates.

Table 2.1: Proportion of children colonized with organisms resistant to listed antibiotics

Antibiotic	***E. coli* (%)**	***Klebsiella* spp. (%)**	**Both *E. coli* and *Klebsiella* spp. (%)**	**Either *E. coli* or *Klebsiella spp.* (%)**
Amoxicillin	52	28	18	62
Amoxicillin-clavulanate	8	8	2	12
Nalidixic acid	36	12	6	42
Ciprofloxacin	24	10	4	30
Gentamicin	6	12	2	16
Amikacin	0	2	0	2
Cefuroxime	26	16	6	36
Cefotaxime	26	16	6	36
Ceftazidime	16	16	4	28
Cefepime	26	16	6	36
Ertapenem	0	2	0	2
Imipenem	0	2	0	2

2.3.2.2 Minimum Inhibitory Concentration

One *K. pneumoniae* isolate (identified from around the ertapenem disc) had reduced zone sizes for imipenem and ertapenem. Carbapenem MICs, determined by gradient diffusion, were 2 µg/ml, 1 µg/ml, and 0.5 µg/ml for ertapenem, imipenem, and meropenem respectively. Based on the 2016 and 2018 CLSI guidelines, the isolate was resistant to ertapenem but susceptible to imipenem and meropenem.

2.4 Discussion

Antibiotic susceptibility testing is vital to assist with choosing appropriate antibiotics, and thus improve the likelihood of success of antibiotic therapy (Kerr, 2005). This chapter described the antibiotic resistance profile of isolates (*E. coli* and *Klebsiella* spp.) obtained from stool samples from healthy children in Cape Town.

The cefpodoxime disc used on the McConkey agar for the selection of putative ESBLs was as effective as the CHROMagar ESBL and can be used as a test to select presumptive ESBL-producing microorganisms (Gazin *et al.*, 2012). A similar study was done by Huang *et al.* using ceftazidime on McConkey agar compared to CHROMagar ESBL, and the specificity was >95% (Huang *et al.*, 2010). The performance of the cefpodoxime against the CHROMagar ESBL is expected since cefpodoxime is currently the drug of choice incorporated in chromogenic media Gazin *et al.* (2012).

Unsurprisingly, *E. coli* was isolated from the stool of the majority of participants. *E. coli* has been reported as one of the most common enteric organisms (Amani *et al.*, 2015; Kebede *et al.*, 2017). Most of the participants were colonized by amoxicillin resistant isolates which is also not surprising, given that amoxicillin is a commonly used agent. All *Klebsiella* isolates were amoxicillin-resistant because *Klebsiella* spp. contains a chromosomally-mediated *bla*SHV-1 (Babini & Livermore, 2000; Chaves *et al.*, 2001).

Of concern is the finding that 42% and 36% of the participants were colonized by quinolone and cephalosporin-resistant isolates, respectively. Colonization by aminoglycoside resistant isolates was also seen in 16% of the participants. Colonization by quinolone-resistant isolates (*E. coli*) has been reported even in the Amazon forest where there is no selection pressure. In that setting, the rates of colonization in children were 45% for nalidixic acid and 15% for ciprofloxacin (Pallecchi *et al.*, 2012). Quinolone resistance in *E. coli* isolates from healthy children in Peru and Bolivia increased between 2002 to 2005, from 35% to 57% for nalidixic acid and 18% to 33% for ciprofloxacin (Bartoloni *et al.*, 2008). Similar to the above-mentioned findings we had 36% and 24% of the participants colonized by nalidixic acid and ciprofloxacin-resistant isolates

respectively. The resistance of *E. coli* and *K. pneumoniae* to ciprofloxacin in South African hospitals ranges from 8-20% and 21-51% respectively (Bamford *et al.*, 2011; Brink *et al.*, 2007). This is somewhat similar to what we have seen in our community isolates, although the data from SA hospitals does not differentiate between community-acquired and hospital-acquired isolates.

A study conducted in a remote area in South America showed that quinolone resistance in *E. coli* was associated with the antimalarial drug chloroquine, which can select for mutations in topoisomerase resulting in quinolone resistance (Davidson *et al.*, 2008). Quinolone resistance has also been associated with certain clones of *E. coli*, which could be a reason for its wide dissemination (Colpan *et al.*, 2013; Johnson *et al.*, 2010; Price *et al.*, 2013).

Previous findings of cephalosporin-resistant *E. coli* isolates colonizing pediatric and adult patients have been documented in Madagascar (22.1%) and Cameroon (54.06%) (Andriatahina *et al.*, 2010; Cantón *et al.*, 2008). However, the colonization rate of 36% from our study was higher than what has been reported in healthy children in the USA (4%) and Tunisia (6.6%) (Ferjani *et al.*, 2017; Islam *et al.*, 2015).

In sub-Saharan Africa and Latin America, rates of resistance to third-generation cephalosporins in Enterobacteriaceae are as high as 46.5% and 87% respectively (CDDEP, 2015; Leopold *et al.*, 2014). Cephalosporin resistance ranged from 39-87% in South African private hospitals (Brink *et al.*, 2007) and 33% of clinical isolates from the public sector in South African hospitals were cephalosporin-resistant (Bamford C, Bonorchis K, Elliott E, 2009) which shows high resistance rates in isolates causing infections. While it is generally recognized that the prevalence of ESBLs is high in isolates from nosocomial settings in South Africa, at up to 58% (Bamford *et al.*, 2009), the prevalence of ESBLs in community settings is thought to be lower. At least 34% of the children in our study carried an ESBL producing organism, which is higher than the 14% global rate of ESBL stool carriage in healthy children and adults reported from WHO-defined regions (Karanika *et al.*, 2016). However, our carriage rate is similar to the overall rate (both hospitalized and healthy individuals) which was reported in Tanzanian children (34.3%). However, in Tanzania, the prevalence rate was 50.4% in pediatric patients and 11.6% in the community

(Tellevik *et al.*, 2016). A previous study conducted in KwaZulu-Natal, South Africa, reported 4.67% fecal ESBL carriage among healthy children in the community (Mahomed & Coovadia, 2015). This is again lower than our findings in the Western Cape. However, our findings are similar to the prevalence of ESBL in *E. coli* and *Klebsiella pneumoniae* from hospitals across Africa which range from 21-33% (Andriatahina *et al.*, 2010; Isendahl *et al.*, 2012; Woerther *et al.*, 2011). The high rate of quinolone and cephalosporin resistance seen in our study supports the hypothesis that the community might be serving as a reservoir of antibiotic resistance which may have originated in nosocomial settings and become established in the community.

The environment has also been reported to be a major reservoir in the dissemination of antibiotic resistance (Marti *et al.*, 2013), and whether these environmental isolates may play a role in humans becoming colonized is yet to be established. The first study of the environmental resistome was published in 2006 (D'Costa, 2006). Morphologically different spore-forming bacteria were collected from the soil in various locations (agricultural, forest and urban) and 480 strains of bacteria were screened for resistance against commonly used antibiotics including natural and synthetic derivatives. Multi-drug resistance was observed in all strains of spore-forming bacteria. This study demonstrated that antibiotic resistance is widely disseminated in environmental bacteria (D'Costa *et al.*, 2006). Grahan *et al* (2010) extracted DNA from agricultural soils in the Netherlands from 1940-2008 and found that antibiotic resistance existed even in the pre-antibiotic era. The *blaCTX-M* gene was also identified in soil prior to its emergence in clinical settings (Knapp *et al.*, 2010).Tap water from Michigan, Ohio, contained bacteria resistant to amoxicillin, ciprofloxacin, gentamicin, rifampicin, and sulfisoxazole (Xi *et al.*, 2009). In another study, *blaTEM* positive isolates in sewage were enriched after treatment (Lachmayr *et al.*, 2009). The *qnrA* gene (associated with quinolone resistance) has been associated with the aquatic bacterium *Shewanella algae* (Chapter 1, subsection 1.5.1.1) (Poirel *et al.*, 2005).

A notable finding in our study was the presence of a single *K. pneumoniae* isolate resistant to carbapenems; this isolate was classified as extensively drug-resistant because it was resistant to all classes of antibiotics tested (Magiorakos *et al.*, 2012). Carbapenem-resistant isolates with similar characteristics have been reported in South Africa (Brink *et al.*, 2008, 2012) but not from

healthy children. At present, all carbapenemase positive Enterobacteriaceae isolates have been referral isolates from both public and private hospitals (Perovic *et al.*, 2016). To the best of our knowledge, no carbapenem-resistant isolates have been reported from the community yet. While the participant from whom this isolate was cultured is a child based in the community, we do not have access to information related to his/her healthcare history, due to the blinded nature of the TB-CHAMP study. If there had been a history of recent hospitalization, for example, this may better explain the concerning presence of this resistant isolate. Though the isolate was resistant to ertapenem and imipenem in the disc diffusion susceptibility testing it was resistant to only ertapenem in the gradient diffusion assay. This might be because the zone diameter of imipenem was 19cm which is exactly the susceptibility breakpoint based on 2016 CLSI guidelines.

Our findings demonstrate a relatively high prevalence (34%) of MDR Enterobacteriaceae in Cape Town. While the sample size is still small and analysis of additional children is ongoing, these resistance rates do highlight the need to better understand the epidemiology of antimicrobial resistance in the community, and to develop interventions (such as antimicrobial stewardship programs) that are focused on the community. Regular surveillance needs to be performed which will provide information to help regulate the use of antibiotics in the community. There may also be a need to implement infection control measures to prevent resistant bacteria from flowing from the community into the hospital.

Chapter 3 : Molecular Resistance Mechanisms and Strain Typing of Isolates collected from Stool Samples

3.1 Introduction

While phenotypic antibiotic susceptibility testing is vital for treatment, it fails to give information on the mechanisms of resistance. This information can help identify mechanisms which are likely to spread to other bacterial species via horizontal gene transfer (Anjum, 2015), and assists with understanding the epidemiology of antimicrobial resistance. Both singleplex and multiplex PCR assays were employed in this chapter to identify acquired resistance genes and to detect mutations responsible for resistance, focusing on resistance to beta-lactams and fluoroquinolones.

Strain typing is a molecular technique which is a necessity in an epidemiological study. The use of strain typing allows us to describe the predominant strains circulating in the community, as well as to describe associations between strain type and presence of resistance. The most common molecular typing techniques used over the years include Restriction Fragment Length Polymorphism (RFLP), Pulsed-Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), Automatic Ribotyping, Repetitive Extragenic Palindromic (rep)-PCR and Multi-Locus Sequence Typing (MLST) (Krawczyk *et al.*, 2016; Ross *et al.*, 2005; Tabit, 2016)

This study makes use of rep-PCR because it is less time consuming, highly reproducible and discriminatory (Castro-Escarpulli *et al.*, 2015). However, it requires specialized skills to analyze the results (Ross *et al.*, 2005). rep-PCR is a DNA fingerprinting technique used to examine strain-specific patterns obtained from PCR amplification of repetitive DNA elements in a bacterial genome. Two major sets of repetitive elements are used for the typing, the repetitive extragenic palindromic (rep) elements which are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem. The palindromic nature of the rep elements and their tendency to form stem-loop structures have led to numerous functions for these highly conserved, dispersed elements (Krawczyk *et al.*, 2016).

This chapter aimed to describe the antibiotic resistance mechanisms and strain diversity of the *E. coli* and *Klebsiella spp*. isolated from stool from healthy children, as described in Chapter 2.

3.2 Materials and Methods

3.2.1 DNA Extraction

A crude DNA extraction method was used to extract DNA from all isolates (n=49) collected as described in chapter 2. Isolates stored in microbank beads (Pro-Lab Diagnostics, Canada) were inoculated on TBA plates (NHLS Media Laboratory, Greenpoint, South Africa) and incubated at 37˚C for 18 hours under aerobic conditions. After incubation, single colonies were resuspended in 400 µl nuclease-free water. The tubes were vortexed to homogenize the solution, and then incubated in a heating block at 95˚C for 30 mins, followed by freezing at -20˚C for 30 mins. Cell debris was sedimented by centrifugation (Sigma 1-15) for 10 mins at 14000 x *g* (Sigma Aldrich, Germany) and the supernatant containing the DNA was collected. Nuclease-free water was used as a negative control and was included in each extraction step. The extracted DNA was stored at -20°C.

3.2.2 Detection of Resistance Genes

3.2.2.1 PCR

PCR was performed on all isolates which were resistant to carbapenems (n=1), cephalosporins (n=21) and quinolones (n=27) to screen for carbapenemase genes, ESBL genes, and quinolone-resistance genes respectively. The multiplex PCRs were performed in a reaction mixture containing 12.5 µl of KAPA2G Multiplex Master mix (KAPA Biosystems, South Africa), 2 µl of template DNA, the relevant primer pairs and water to make up a final volume of 25µl. KAPA ReadyMix (KAPA Biosystems) was used for the singleplex PCRs, except for SHV for which KAPA2G Multiplex Master mix was used. The primer sequences and concentrations used in each reaction are described in Table 3.1. *rpoB* was used as an internal control in all the multiplex PCR reactions except for the assays targeting the *qnr* genes, as the *rpoB* primers interfered with the amplification of the *qnrB* gene.

3.2.2.2 Gel Electrophoresis

All amplicons were separated on 2% Seakem LE agarose (Lonza, South Africa) gel in 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer at 120 volts for 50 mins except for *qnr* genes which were separated for 90 mins. The band sizes were determined using New Biolabs 100bp Ladder (New England Biolabs Inc.). The amplicons were visualized under UV light using the UVitec Cambridge Alliance 2 gel documentation system.

Table 3.1: Primer Sequences for PCR

Target	primers	Concentration of primer	Sequences (5' – 3')	Size (bp)	References
qnrA	qnrA-F	0.2 µM	TCAGCAAGAGGATTTCTCA	627	Liu *et al.*, 2012
	qnrA-R		GGCAGCACTATTACTCCCA		
qnrB	qnrB-F	0.2 µM	GATCGTGAAAGCCAGAAAGG	469	Liu *et al.*, 2012
	qnrB-R		ACGATGCCTGGTAGTTGTCC		
qnrS	qnrS-F	0.2 µM	ACGACATTCGTCAACTGCAA	417	Liu *et al.*, 2012
	qnrS-R		TAAATTGGCACCCTGTAGGC		
gyrA	gyrA-F	0.2 µM	ACGTACTAGGCAATGACTGG	191	Liu *et al.*, 2012
	gyrA-R		AGAAGTCGCCGTCGATAGAA		
parC	ParC-F	0.2 µM	TGTATGCGATGTCTGAACTG	264	Liu *et al.*, 2012
	ParC-R		CTCAATAGCAGCTCGGAATA		
*bla*CTX-M	CTX-F	0.2 µM	ATGTGCAGYACCAGTAARGTKATGGC	593	Monstein *et al.*, 2007
	CTX-R		TGGGTRAARTARGTSACCAGAAYCAGCGG		
*bla*SHV	SHV-F	0.24 µM	ATGCGTTATATTCGCCTGTG	747	Monstein *et al.*, 2007
	SHV-R		TGCTTTGTTATTCGGGCCAA		
*bla*TEM	TEM-F	0.2 µM	TCGCCGCATACACTATTCTCAGAATGA	445	Monstein *et al.*, 2007
	TEM-R		ACGCTCACCGGCTCCAGATTTAT		
*bla*VIM	VIM-F	0.2 µM	GATGGTGTTTGGTCGCATA	390	Zowawi *et al.*, 2014
	VIM-R		CGAATGCGCAGCACCAG		
*bla*IMP	IMP-F	0.2 µM	CTACCGCAGCAGAGTCTTTGC	591	Zowawi *et al.*, 2014
	IMP-R		GAACAACCAGTTTTGCCTTACC		
*bla*KPC	KPC-F	0.2 µM	ATCTGACAACAGGCATGACG	452	Zowawi *et al.*, 2014
	KPC-R		GACGGCCAACACAATAGGTG		
*bla*OXA	OXA-F	0.2 µM	GCGTGGTTAAGGATGAACAC	438	Zowawi *et al.*, 2014
	OXA-R		CATCAAGTTCAACCCAACCG		
*bla*NDM	NDM-F	0.2 µM	GCAGGTTGATCTCCTGCTTG	203	Zowawi *et al.*, 2014
	NDM-R		ACGGTTTGGCGATCTGGT		
*bla*GES	GES-F	0.2 µM	GTTTTGCAATGTGCTCAACG	371	Weldhagen and Prinsloo, 2004
	GES-R		TGCCATAGCAATAGGCGTAG		
rpoB	rpoB-F	ESBL PCR assays: 0.2 µM. Carbapenemase PCR assays: 0.08 µM	AACCAGTTCCGCGTTGGCCTGG	1088	(Hoffmann & Roggenkamp 2003)
	rpoB-R		CCTGAACAACACGCTCGGA		

3.2.2.3 Control Strains

*bla*TEM, *bla*SHV and blaCTX-M, *qnrB* and *qnrS* positive controls were obtained from a previous study in the department (Table 3.2), where isolates with *qnrB* and *qnrS* had been identified by analysis of whole genome sequences. The amplification products of these controls had been previously confirmed by Sanger sequencing. Unfortunately, a *qnrA* positive control was not included in this assay, as we were unable to source any positive controls from South African collaborators, or overseas collaborators in Freiburg, Wurzburg, Cardiff or Nairobi. *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603 were used as positive controls for *parC* and *gyrA* singleplex PCR. (Table 3.2).

Table 3.2: Positive controls used for PCR

POSTIVE CONTROLS		**SOURCE**
Carbapenemase controls		National Institute for Communicable Diseases (NICD, South Africa) satellite unit at Groote Schuur Hospital
*bla*IMP	*K. pneumoniae*	
*bla*OXA	*K. pneumoniae*	
*bla*VIM	*K. pneumoniae*	
*bla*GES	*K. pneumoniae*	
*bla*NDM	*K. pneumoniae*	
*bla*KPC	*K. pneumoniae*	
ESBL controls		A previous study in the Department of Medical Microbiology, Stellenbosch University.
*bla*SHV	Single *E. coli* isolate positive for all three genes	
*bla*TEM		
*bla*CTX-M		
***qnr* controls**		
qnrA	NA	
qnrB	Single *E. coli* isolate positive for both genes	
qnrS		
Detection of mutations		National Health Laboratory Service (NHLS) diagnostic laboratory at Tygerberg Hospital (TBH)
gyrA	*E. coli* ATCC25922 *K. pneumoniae* ATCC700603	
parC		

3.2.2.4 Carbapenemase Genes

Isolates with reduced susceptibility to carbapenems were subjected to a touchdown multiplex PCR assay to differentiate 6 carbapenemase genes – *bla*VIM, *bla*IMP, *bla*GES, *bla*KPC, *bla*OXA and *bla*NDM (Weldhagen & Prinsloo, 2004; Zowawi *et al.*, 2014). The multiplex PCR was split in two reactions: *bla*VIM, *bla*IMP, *bla*OXA and *bla*GES, *bla*KPC, *bla*NDM (Table 3.3), because the band sizes of *bla*KPC and *bla*OXA differ by only 14 bp which would be difficult to differentiate on a gel image from a single assay.

Table 3.3: Cycling conditions for the carbapenemase touch down multiplex PCR

	Temperature(˚C)	**Time**	**No. of cycles**
Initial denaturing	95	3min	1
Denaturing	95	15sec	20
Annealing	68	30sec	
Extension	72	1min	
Denaturing	95	15sec	10
Annealing	62	30sec	
Extension	72	1min	
Final Extension	72	5min	1
Hold	4	∞	

3.2.2.5 Extended-spectrum β-lactamase genes

Extended-spectrum beta-lactamase genes from phenotypically cephalosporin resistant *E. coli* and *Klebsiella* isolates were characterized using a multiplex PCR assay that targets the *bla*CTX-M, *bla*SHV and *bla*TEM families (Monstein *et al.*, 2007) (Table 3.4).

Table 3.4: Cycling conditions for the ESBL multiplex PCR

	Temperature(˚C)	Time	No. of cycles
Initial denaturing	95	3min	1
Denaturing		15sec	35
Annealing	67	30sec	
Extension	72	30sec	
Final Extension		3min	1
Hold	4	∞	

Molecular Classification of blaTEM and blaSHV Genes by DNA Sequence Analysis

All variants of *bla*CTX-M have the extended spectrum activity characteristic of ESBLs, but many *bla*SHV and *bla*TEM variants do not have this extended-spectrum activity. All *bla*TEM and *bla*SHV positive isolates were subjected to a singleplex PCR (for either *bla*TEM, or *bla*SHV, or both, as appropriate), followed by Sanger sequencing to differentiate between genes encoding enzymes with and without extended spectrum activity. Fifteen to twenty microlitres of the amplicons were sent for Sanger sequencing at Inqaba BioTech (South Africa), using the forward PCR primers. The sequencing was performed using BrilliantDye^{TM} Terminator V3.1.

The nucleotide sequences were edited and analyzed using BioEdit Sequence Alignment Editor version 7.2.5. *bla*SHV sequences were converted to amino acid sequences and compared to *bla*SHV protein sequences because the DNA sequences have multiple synonymous nucleotide substitutions, which made the nucleotide sequences difficult to interpret.

3.2.2.6 Quinolone Resistance Mechanisms

qnr genes

Quinolone-resistant isolates (resistant to nalidixic acid or ciprofloxacin) were analyzed for the presence of *qnr* genes (*qnrA, qnrB,* and *qnrS)* using a multiplex PCR (Liu, Liao, *et al.*, 2012), using the cycling conditions described in table 3.5.

Table 3.5: Cycling conditions for the *qnr* multiplex PCR and singleplex *parC* and *gyrA* PCRs

	Temperature(˚C)	**Time**	**No. of cycles**
Initial denaturing	95	3min	1
Denaturing		15sec	35
Annealing	64 (qnr) 60 (gyrA; parC)	30sec	
Extension	72	30sec	
Final Extension		3min	1
Hold	4	∞	

parC and gyrA Mutations

Singleplex PCR was used to amplify the *gyrA* and *parC* genes from all quinolone-resistant isolates (Liu, Liao, *et al.*, 2012), as shown in Table 3.5. The PCR products were sequenced at Inqaba BioTech. The initial set of PCR products were sequenced using both PCR primers, but since the forward sequence covered the positions of all the expected mutations, the remaining products were sequenced using only the forward primer. BioEdit software was used to align and detect mutations on the *gyrA* and *parC* loci. The *gyrA* and *parC* sequences were compared with respective wild-type sequences from NCBI with accession number NC_000913.

3.2.3 Repetitive Palindromic Sequence (rep)-PCR

All isolates were subjected to rep-PCR (Versalovic *et al.*, 1991) to generate a specific DNA pattern or fingerprint to determine the genetic relatedness of the isolates; including available matched susceptible and resistant isolates. Based on Higgins *et al*, (2012) classification method, similarity of 95% or above would indicate relatedness, while unrelated strains had similarity of <95%.

The primers used for the rep-PCR include; REP-1-F 5′- ICGICTTATCIGGCCTAC-3′ and REP-2-R: 5′-CGGICTACIGCIGCIIII-3′ as previously described by Versalovic et al., (1991). The PCR was performed in a reaction mixture containing 12.5 µl of KAPA2G Multiplex Master mix,

2 µl of template DNA, the primer pairs (REP-1-F & REP-2-R) and water to make up a final volume of 25 µl. The concentration of each primer used was 0.2 µM; and the cycling conditions are described in Table 3.6.

Table 3.6: Cycling conditions for rep-PCR

	Temperature(˚C)	Time	No. of cycles
Initial denaturing	95	5min	1
Denaturing	95	30sec	30
Annealing	40	30sec	
Extension	72	8min	
Final Extension	72	16min	1
hold	4	∞	

Amplicons were separated on 2% Seakem LE agarose gel in 1X TAE buffer at 80 volts for 84 mins. The band sizes were determined using DirectLoad™ Wide Range DNA Marker *(*Sigma, Aldrich, Germany). The amplicons were visualized as previously described. The rep-PCR gel image was incorporated into GelComparII Bionumerics (version 7.5) and a phylogenetic tree was generated using the dice-band approach with the UPGMA algorithm. Isolates with 95% and above similarity were considered related.

3.3 Results

3.3.1 Identification of Carbapenemase Genes

Multiplex PCR showed that the single carbapenem-resistant *K. pneumoniae* isolate (K4) harbored the *bla*NDM carbapenemase gene (Figure 3.1)

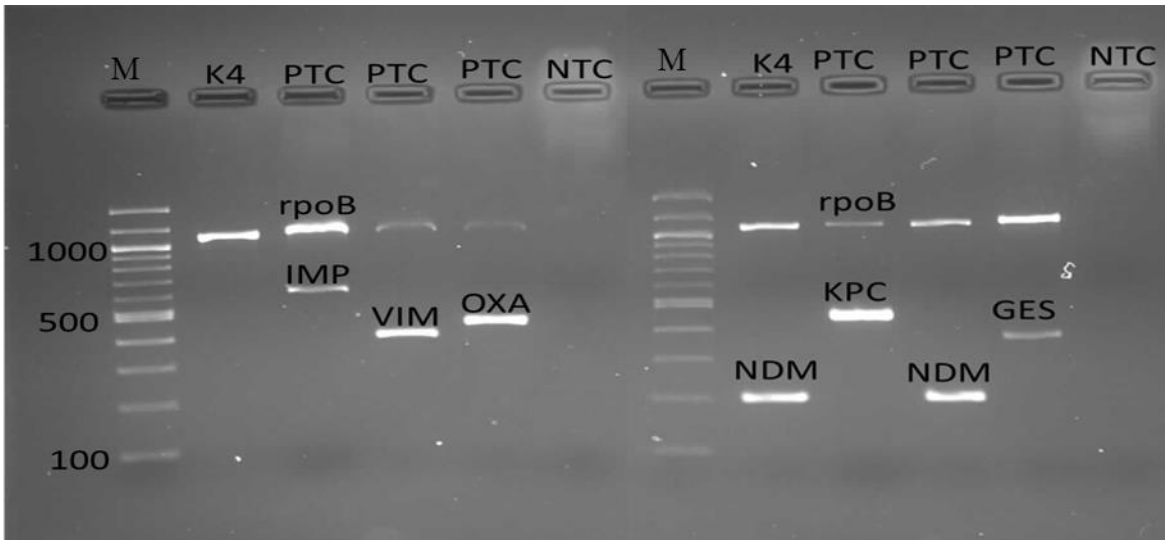


Figure 3.1. **Carbapenemase Multiplex PCR.** 2% agarose gel showing common carbapenemase genes *bla*IMP, *bla*VIM, *bla*OXA (left side of gel) and *bla*NDM, *bla*KPC and *bla*GES (right side of gel), with an internal control (*rpoB* gene). **M** = Kapa Universal DNA Marker, **PTC**= Positive Template Control and **NTC**= No Template Control. **K4** = *Klebsiella* isolate.

3.3.2 Identification and Characterization of ESBL Genes

Thirteen *E. coli* and 8 *Klebsiella* spp. isolates were resistant to cephalosporins and therefore predicted to be ESBL producers. Multiplex PCR was used to identify the three most common ESBL gene families; *bla*SHV, *bla*CTX-M, and *bla*TEM. An example of one of the agarose gels showing the presence of different ESBL-related genes in a selection of isolates is shown in Figure 3.2 with the internal control, *rpoB*, present in all isolates. All thirteen (100%) of the cephalosporin resistant *E. coli* isolates harbored a *bla*CTX-M gene and 7 (54%) had an additional *bla*TEM gene. However, none of the *E. coli* isolates harbored the *bla*SHV gene.

*bla*SHV was detected in all 8 (100%) of the cephalosporin resistant *Klebsiella* isolates of which 7 (88%) also harbored *bla*CTX-M and *bla*TEM genes (Figure 3.3).

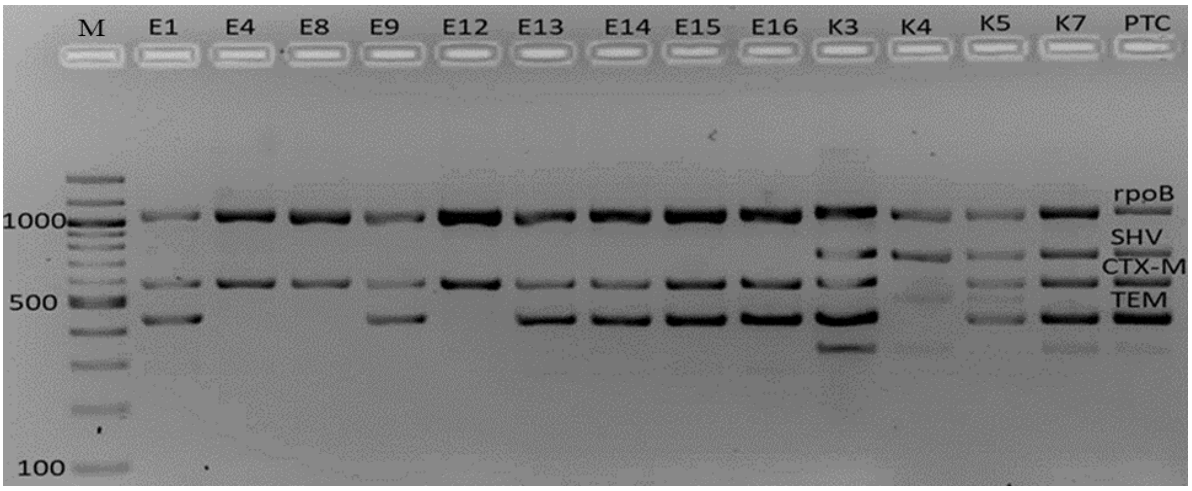


Figure 3.2: **ESBL Multiplex PCR.** 2% agarose gel showing ESBL family genes (*bla*CTX-M, *bla*TEM, and *bla*SHV) with an internal control (*rpoB*) gene. **M** = KAPA Universal DNA Ladder. **PTC** = Positive Template Control. **K** = *Klebsiella* isolates and **E** = *E. coli* isolates.

Figure 3.3: Distribution of *bla*CTXM, *bla*TEM and *bla*SHV genes in cephalosporin-resistant *E. coli* isolates (A) and *Klebsiella spp*.(B).

All *bla*TEM genes were identified as *bla*TEM-1 based on 99.2% DNA sequence identity to a published *bla*TEM-1 sequence (NC_022885.) over 45-47% of the *bla*TEM-1 gene. Therefore, all *bla*TEM genes were identified as β-lactamases without extended spectrum activity.

Three of the *bla*SHV genes were identified as *bla*SHV-1, four were identified as *bla*SHV-11 and one was identified as *bla*SHV-26 based on 100% amino acid identity to published *bla*SHV-1 (DQ478720.), *bla*SHV-11 (NC 019157.), and *bla*SHV-26 (AF227204.) respectively, with 79.7% coverage. Therefore, all the *bla*SHV genes were identified as β-lactamases without extended spectrum activity. The one *Klebsiella* isolate with only *bla*SHV and no *bla*CTX-M or *bla*TEM (Fig 3.3) was the isolate with the *bla*NDM carbapenemase.

Eighteen (36%) of the children were colonized by one or more ESBL-producing isolates, all of which were *bla*CTX-M related.

3.3.3 Quinolone Resistance Genes

Plasmid-mediated quinolone resistance genes

Of the 21 quinolone-resistant *E. coli* isolates, 5 (24%) harbored *qnrS* while of the 6 quinolone-resistant *Klebsiella* isolates, 4 (67%) had *qnrB.* Overall, 8 (16%) of the participants were colonized by isolates with plasmid-mediated quinolone resistance genes (Table 3.7). Two (4%) of the participants were colonized by *E. coli* isolates with both PMQR genes and ESBL genes while 4 (8%) were colonized by *Klebsiella* isolates with both PMQR genes and ESBL genes.

Chromosomal Mutations

All of the quinolone-resistant isolates had at least a single mutation in either the *parC* or *gyrA* genes. The most common mutation in *E. coli* was S83L in *gyrA* and it was often (62%) coupled with a D87N mutation in *gyrA* (Table 3.7). Seven *E. coli* isolates had only a single mutation in *gyrA* (S83L in six; D87W in one); all seven of these isolates were resistant to nalidixic acid but ciprofloxacin susceptible. All isolates with at least two mutations (or a single mutation plus a *qnr*-gene) were resistant to both nalidixic acid and ciprofloxacin.

All quinolone resistant *Klebsiella* isolates, except K4, had only double *parC* mutations, S129A and A141V, and no mutation in *gyrA* (Table 3.8). Isolate K4 (the only carbapenem-resistant

isolate) had three mutations (S80I, S129A, and A141V) in *parC* and a double mutation in *gyrA* (D87A and S83L). All *Klebsiella* isolates were resistant to both nalidixic acid and ciprofloxacin. All isolates with plasmid-mediated quinolone resistance also had mutations in either *parC* or *gyrA.*

Table 3.7: Mechanisms of resistance in quinolone-resistant *E. coli* isolates

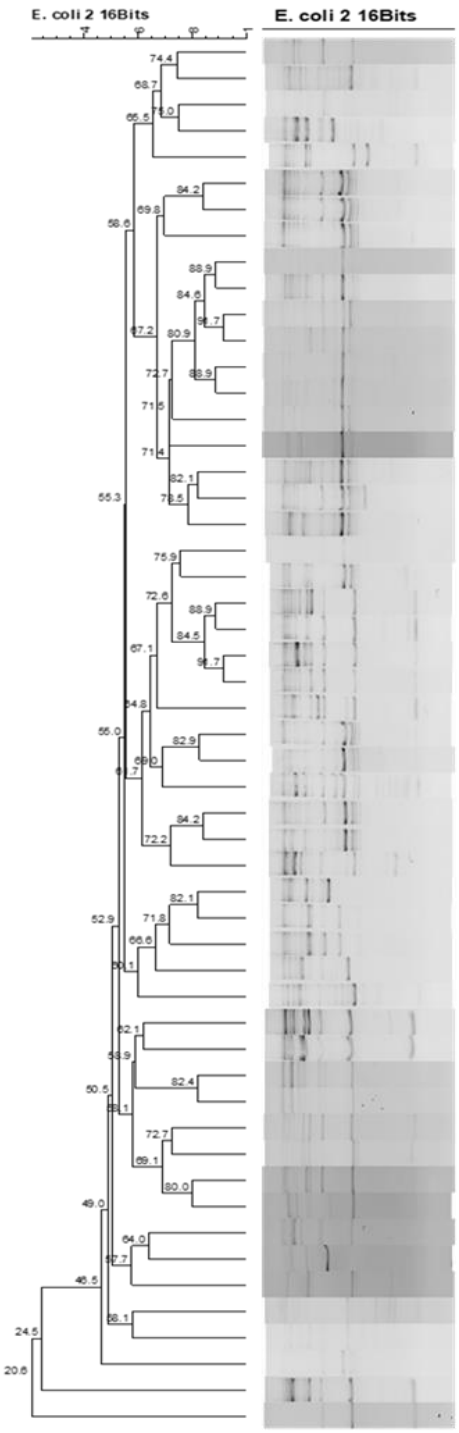
***E. coli* isolate**	**Quinolone susceptibility**		**Quinolone resistance gene and codon**					
	Nalidixic Acid	**Ciprofloxacin**	**PMQR**		***gyrA***		***parC***	
gene/codon			***qnrB***	***qnrS***	**83**	**87**	**80**	**84**
E. 1	R	S			S83L	WT	WT	WT
E. 2	R	R			S83L	D87N	S80I	WT
E. 3	R	S			S83L	WT	WT	WT
E. 4	R	R			S83L	D87N	S80I	E84V
E. 5	R	R		X	S83L	D87N	S80R	WT
E. 6	R	R			S83L	D87N	S80I	WT
E. 7	R	R		X	S83L	D87N	S80R	WT
E. 8	R	S			S83L	WT	WT	WT
E. 9	R	R			S83L	D87N	S80I	E84V
E. 10	R	S			WT	D87W	WT	WT
E. 11	R	R		X	S83L	D87N	S80I	WT
E. 12	R	R			S83L	D87N	S80I	WT
E. 13	R	R		X	S83L	D87N	S80I	WT
E. 14	R	R			S83L	D87N	S80I	WT
E.17	R	R			S83L	D87N	S80I	WT
E. 19	R	S			S83L	WT	WT	WT
E. 20	R	R		X	S83L	WT	WT	WT
E. 21	R	R			S83L	D87N	S80I	WT
E. 22	R	S			S83L	WT	WT	WT
E. 23	R	R			S83L	D87N	S80I	WT
E. 24	R	S			S83L	WT	WT	WT

Table 3.8: Mechanisms of resistance in quinolone-resistant *Klebsiella* isolates

Klebsiella isolates									
Isolate ID	**Nalidixic Acid**	**Ciprofloxacin**	**PMQR**		***parC***			***gyrA***	
gene/codon			*qnrB*	*qnrS*	80	129	141	83	87
K. 1	R	R			WT	S129A	A141V	WT	WT
K.2	R	R			WT	S129A	A141V	WT	WT
K. 3	R	R	X		WT	S129A	A141V	WT	WT
K. 4	R	R	X		S80I	S129A	A141V	S83L	D87A
K. 5	R	R	X		WT	S129A	A141V	WT	WT
K. 7	R	R	X		WT	S129A	A141V	WT	WT

3.3.4 Strain Typing

rep-PCR was performed to determine the relatedness of the isolates. Since the culture and selection aimed to collect multiple isolates from the same individual, the typing included isolates from the same stool sample. There were 13 pairs of *E. coli* isolates and 8 pairs of *Klebsiella* isolates from the same sample. Each of the isolates within a pair was also unrelated, with the similarity between the isolates and individual pairs ranging from 20.6 to 92.6% (Figure 3.4 & 3.3).



Isolate	ESBL'S	PMQR	gyrA	parC
E8	CTX-M		S83L	
E4	CTX-M		D87N,S83L	S80I, E84V
E26				
E34				
E35				
E5		qnrS	D87N,S83L	S80R
E7		qnrS	D87N,S83L	S80R
E33				
E14	CTX-M, TEM		D87N,S83L	S80I
E28				
E46				
E47				
E13	CTX-M, TEM	qnrS	D87N,S83L	S80I
E17			D87N,S83L	
E10			D87N	
E19	CTX-M, TEM			
E30				
E32				
E31				
E27				
E43				
E38				
E21	CTX-M		D87N,S83L	S80I
E9	TEM, CTX-M		D87N,S83L	S80I, E84V
E39				
E45				
E24			S83L	
E2			D87N,S83L	S80I
E52				
E37				
E22			S83L	
E23			D87N,S83L	S80I
E41				
E44				
E40				
E42				
E36				
E3			S83L	
E6			D87N,S83L	S80I
E16	CTX-M, TEM			
E15	CTX-M, TEM			
E12	CTX-M		D87N,S83L	S80I
E48				
E49				
E20	CTX-M	qnrS		
E18	CTX-M			
E51				
E50				
ATCC 25.				
E1	TEM, CTX-M		S83L	S80I
E25				
E29				
E11		qnrS	D87N,S83L	S80I

Figure 3.2: Genetic relatedness of *E. coli* isolates

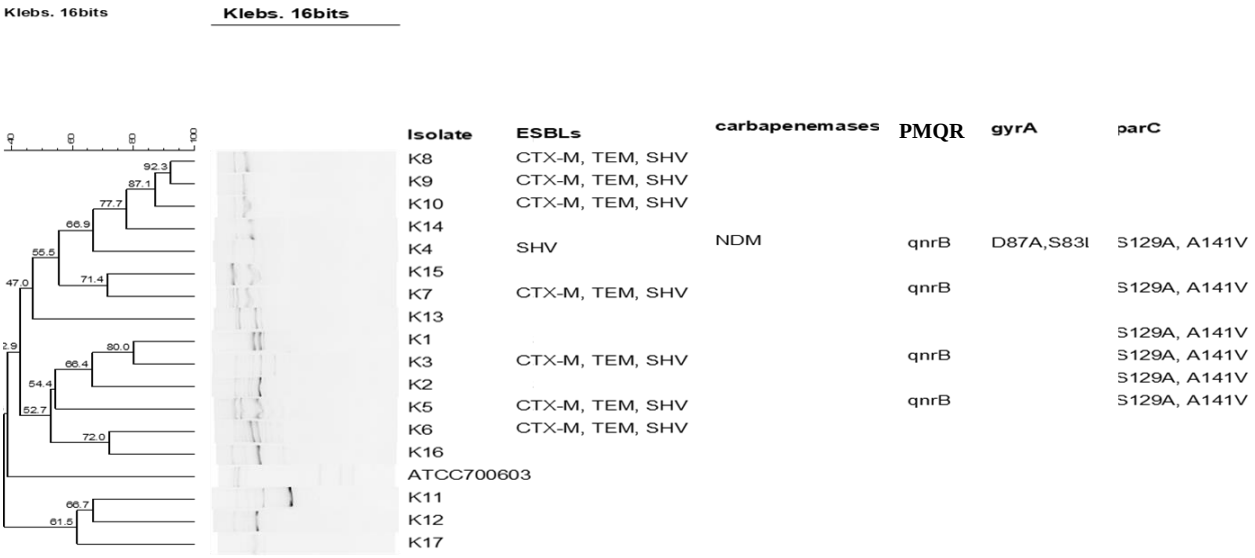


Figure 3.3: Genetic relatedness of *Klebsiella* isolates

3.5 Discussion

While mutations in chromosomal genes (such as *gyrA* and *parC*) are a well-described cause of resistance (Woodford & Ellington, 2007), plasmid-mediated resistance genes are of greater concern because these mobile genetic elements are likely to be transferred between bacterial species. Plasmid-mediated antibiotic resistance genes are widely disseminated and a global threat (Burmeister, 2015). Plasmids harbor genes which have many functions, including virulence factors and antibiotic resistance, that help the bacteria to survive in their environment and to withstand the antibiotics that are used to treat them (Ramirez *et al.*, 2015).

One carbapenem-resistant *Klebsiella* isolate (K4) harbored the *bla*NDM carbapenemase gene. Though this isolate was resistant to cephalosporins, it had only *bla*SHV-1 (non-ESBL), suggesting that the *bla*NDM gene conferred cephalosporin resistance. The first *bla*NDM positive isolate described in South Africa was resistant to all antibiotics except colistin and tigecycline which is a peculiar susceptibility profile for the *bla*NDM gene (Lowman *et al.*, 2011) but is similar to our findings. *bla*NDM-positive isolates with similar susceptibility profiles have been reported in Pakistan, India and the UK (Kumarasamy *et al.*, 2010).

The most common ESBL gene was *bla*CTX-M in both *E. coli* and *Klebsiella* isolates with an overall rate of 94% among the predicted ESBL-producing isolates. Only one (K4) of the ESBL-producing *Klebsiella* isolates did not harbor *bla*CTX-M. Interestingly, this isolate contained a *bla*SHV-1 gene which is not an ESBL. The isolate was phenotypically resistant to cephalosporins, which might be attributed to the presence of *bla*NDM as described previously. All the other *bla*SHV (*bla*SHV-1, *bla*SHV-11, and *bla*SHV-26) and *bla*TEM (*bla*TEM-1) genes detected by PCR in our isolates lack extended spectrum activity and are not regarded as ESBLs (Barguigua, El Otmani, *et al.*, 2013).

The predominance of *bla*CTX-M related ESBLs is consistent with global findings. A previous study in Cambodia and Cameroon reported 100% of ESBL-producing *E.coli* from clinical isolates harbored *bla*CTX-M (Lonchel *et al.*, 2012; Ruppé *et al.*, 2009). In healthy children from

Tunisia, 6 out of 7 ESBL-producing *E. coli* harbored *bla*CTX-M (Ferjani *et al.*, 2017). The prevalence of *bla*CTX-M in ESBL-producing clinical Enterobacteriaceae isolates was 90% and 94.7% in Canada and Tanzania respectively (Chaubey *et al.*, 2010; Tellevik *et al.*, 2016). In South Africa, 95% of ESBL-producing *E. coli* recovered from urine and pus swabs from community hospitals harbored *bla*CTX-M in both children and adults (Peirano & Pitout, 2010).

Two out of 5 *qnrS*-positive *E. coli* isolates harbored *bla*CTX-M whilst 3 out of 4 *qnrB*-positive *Klebsiella* isolates had *bla*CTX-M. *qnr* genes are known to co-exist with ESBLs because they are often located on the same plasmids (Crémet *et al.*, 2011). Interestingly the only PMQR gene detected in our *E. coli* isolates was *qnrS*, while in *Klebsiella* isolates, we only detected *qnrB*. In Iran, a predominance of *qnrS* and *qnrB* has been reported in *E. coli* and *Klebsiella* isolates respectively (Mirzaii *et al.*, 2018). *qnr* genes, by themselves, do not confer quinolone resistance, but promote the selection of higher-levels of resistance, increasing the effect of *parC* and *gyrA* mutations on quinolone MICs (discussed in chapter 1, section 1.5.1).

Mutations in the *parC* and *gyrA* genes are well known to cause resistance to quinolones. All quinolone-resistant isolates had at least one mutation in either the *parC* or *gyrA* genes. *E. coli* isolates with mutations in both *gyrA* and *parC* were resistant to nalidixic acid and ciprofloxacin while those with mutations in only *gyrA* were resistant to only nalidixic acid. A similar finding has been reported by Liu *et al*, (2012) and Nouri *et al*, (2016). In previous studies, eighty-three of 92 isolates with at least three point mutations showed ciprofloxacin MICs of 32 μg/ml while all isolates with a single point mutation in *gyrA*, were not resistant to ciprofloxacin (MICs ranged from 0.25 to 2 μg/ml) (Onseedaeng & Ratthawongjirakul, 2016). However, one *E. coli* isolate from our study had a single mutation in *gyrA* as well as the *qnrS* gene and was resistant to nalidixic acid and ciprofloxacin, which supports the hypothesis that PMQR genes promote the selection of higher-level resistance (Guan *et al.*, 2013). *E. coli* isolates with double mutations in *gyrA* had at least one mutation in *parC*, while there were no isolates with a single mutation in *gyrA* and a mutation in *parC*. This may support the hypothesis that *gyrA* appears as a primary fluoroquinolone target in Enterobacteriaceae (Piekarska *et al.*, 2015).

Sixty-two percent of the quinolone-resistant *E. coli* isolates had the double S83L and D87N mutations in *gyrA*. A similar mutation pattern was reported in healthy children from the Amazon forest (Pallecchi *et al.*, 2012). The S80I mutation in *parC*, seen among 43% of our isolates, has also been reported by Onseedaeg et al, (2016) in clinical isolates.

Amongst the *Klebsiella* isolates, only one isolate had a mutation in *gyrA* (the single carbapenem-resistant isolate), while in the *parC* genes the most common mutations were S129A and A141V, similar to what has been reported by Nouri et al, (2016) and Kao *et al*, (2016) in *Pseudomonas aeruginosa* and *E. coli* respectively. The *Klebsiella* isolates with mutations in only the *parC* gene were resistant to ciprofloxacin. This is in line with what Liu *et al* reported in 2012, namely that mutations in *parC* alone lead to higher ciprofloxacin MICs in *E. coli* isolates. However, the mutations occurred at position S80I and the MICs ranged from 0.125 to 128 $mg.l^{-1}$ (Liu, Liao, *et al.*, 2012). Almost all the *Klebsiella* isolates with mutations in only *parC* and that were resistant to ciprofloxacin harbored PMQR genes; this may also support the hypothesis mentioned earlier. However, one isolate (K1) had double mutations in *parC* (S129A and A141V) and no PMQR gene detected but was resistant to ciprofloxacin.

Results from the rep-PCR show that the *E. coli* and *Klebsiella* isolates from the stool of the participants were unrelated, ESBL-producing and quinolone-resistant isolates were not associated with a particular lineage. This is not surprising given the great microbial diversity to be expected in stool samples and given that the participants are not epidemiologically related. Interestingly, even isolates from within the same patient sample were unrelated, highlighting the existence of diversity within a patient sample. This finding confirmed what has been published by Schlager *et al.*, (2002), showing that the normal intestinal flora is diverse and the composition changes over time. Similar findings have also been published by Caugant *et al.*, (1981). Factors such as diet and lifestyle have been reported to influence variations in stool microbiota (Lozupone *et al.*, 2012; Senghor *et al.*, 2018)

Our findings report a high prevalence of plasmid-mediated quinolone and cephalosporin resistance genes in *E. coli* and *Klebsiella* isolates in children in Cape Town. A single carbapenem-resistant isolate harboring *bla*NDM was also detected, which is concerning. Coexistence of two or more resistance genes has also been recorded in our study. This increases

the risk of the children being infected by resistant isolates since plasmid-mediated resistance can disseminate among bacteria. Appropriate measures such as hand-washing and personal hygiene need to be encouraged in schools and homes to reduce the risk of infections, and the need to use antibiotics judiciously must be emphasized.

Chapter 4 : Exposure of Stool Samples to Sub-clinical Concentrations of Selected Antibiotics

4.1 Introduction

The indiscriminate and inappropriate use of antibiotics in humans, animals and the environment (Andersson & Hughes, 2014) results in regular exposure of bacteria (including those found as normal human flora) to varying (and sometimes sub-lethal) concentrations of antibacterial agents (Baquero & Negri, 1997). These low-level concentrations may stimulate the emergence of low-level resistance which can serve as a pacesetter for high-level resistance (Baquero, 2001).

Studies that have examined the effect of *in-vitro* exposure of organisms to antibiotics have improved our understanding of resistant microorganisms (Gullberg et al. 2011; Sandegren 2014; Andersson & Hughes 2014). Studies investigating *in-vitro* exposure have shown a link with the emergence of resistance in both microbial populations and sub-populations (Cantón & Morosini, 2011; Gullberg *et al.*, 2011; Sandegren, 2014). Understanding the association between antibiotic exposure and development of resistance is important to better understand the implications of antimicrobial use, and potentially to design treatment regimens that may reduce the emergence of resistance (Baquero, 2001).

Sub-MIC levels of β-lactams and fluoroquinolones have been documented to select for and enrich resistance (Couce & Blázquez, 2009) (chapter 1, section 1.6). Quinolones have been reported to have the highest recovery rate in stool samples (De Lastours *et al.*, 2014). Since the TB-CHAMP study is evaluating the efficacy of levofloxacin for MDR-TB prophylaxis (Tb-Champ protocol, 2015)(Tb-Champ protocol, 2015) it creates an opportunity to do an *in-vitro* pilot study to examine the effect of the intervention on the carriage of resistant organisms in the participants.

Most *in vitro* antibiotic exposure studies differ in the duration of exposure, classes of antibiotics used and most if not all use bacterial isolates and not complex human samples (Cantón & Morosini, 2011; Olofsson & Cars, 2007). This chapter aims to investigate the effects of antibiotic exposure on the transfer and acquisition of resistance in stool using an *in vitro* model. As a novel

approach was used in this study, the practically of the model was evaluated and used to advise future studies.

4.2 Materials and Methods

4.2.1 Antibiotic Exposure

This phase of the study aimed to mimic the exposure of organisms in stool samples to sub-clinical concentrations of antibiotics (as may occur when antibiotics are administered systemically) and to describe how this influences the carriage and development of resistance in organisms in the stool sample. The antibiotics chosen were amoxicillin and ciprofloxacin (as two commonly used oral agents), and colistin. While colistin is not commonly used and is only administered intravenously to humans, it is sometimes added to animal feed. Given its importance as a (human) antibiotic of last resort, and with emerging reports of colistin resistance, we included it in this pilot study to potentially inform future studies that would more appropriately focus on its effects in animals.

For the purposes of this pilot, the first 10 stool samples received were used. One hundred micrograms (100 µg) of each of these 10 baseline stool samples was resuspended in 10 ml of saline to make up a final concentration of 10 mg/ml. One thousand microliters (1000 µl) of the stool suspension was aliquoted into four 20 ml sterile vials, each vial containing 1000 µl of one of the three selected antibiotics, as well as one vial with no antibiotic. After adding the stool sample, the final concentration of each antibiotic in the stool suspension was as follows:

- amoxicillin: 8µg/ml and 2µg/ml
- ciprofloxacin: 1µg/ml and 0.25µg/ml
- colistin: 2µg/ml and 0.5µg/ml

These concentrations were chosen as they represent a one-fold and four-fold reduction compared to the susceptible breakpoint concentration for each agent, based on CLSI guidelines. For purposes of clarity, these will be referred to as the “high concentration” and “low concentration” exposures (Figure 4.1 a & b).

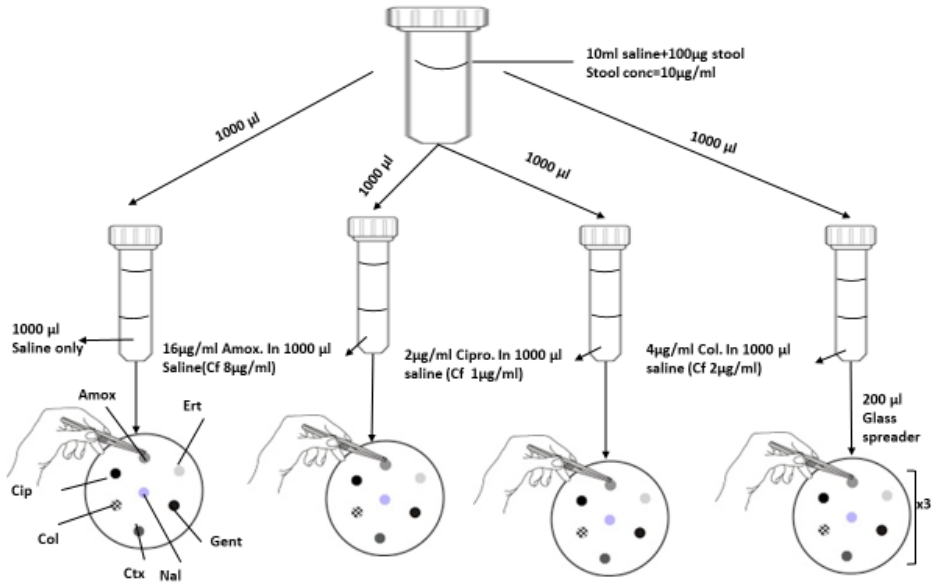


Figure 4.1a: Set-up for the "high concentration" antibiotic exposure experiment. **Cf**= Final Concentration, **Amox**= amoxicillin, **Ert**= ertapenem, **Gent**= gentamicin, **Nal**= nalidixic acid, **Ctx**= Cefotaxime, **Col**= colistin, **Cip**= ciprofloxacin

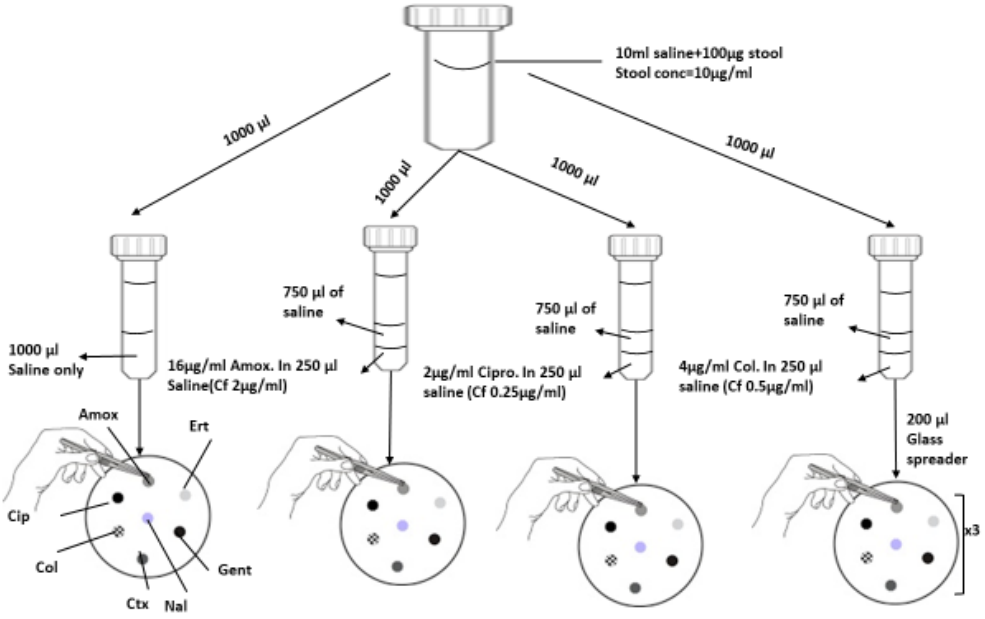


Figure 4.1b: Set-up for the "low concentration" antibiotic exposure experiment. **Cf**= Final Concentration, **Amox**= amoxicillin, **Ert**= ertapenem, **Gent**= gentamicin, **Nal**= nalidixic acid, **Ctx**= Cefotaxime, **Col**= colistin, **Cip**= ciprofloxacin.

The sterile vials were incubated under aerobic conditions for 48 hours at 37˚C, following which a 200 µl aliquot was spread onto the surface of a MacConkey agar plate with crystal violet (NHLS Media Laboratory, Greenpoint, South Africa) to suppress the growth of Gram-positive organisms, using a glass spreader. Antibiotic discs [amoxicillin (10 µg), ertapenem (10 µg), ciprofloxacin (5 µg), colistin (10 µg), cefotaxime (30 µg) and nalidixic acid (30 µg)] (Mast, Laboratories, UK) were added (Figure 4.1a & b), and three replicates were performed. The agar plates were incubated for 18-24 hours at 37°C. Isolates morphologically consistent with *E. coli* and *K. pneumoniae* growing inside the zones of inhibition of the various antibiotic discs were enumerated using a semi-quantitative approach. The following categories were used to describe the growth of Gram-negative bacilli around the antibiotic discs in the control (no antibiotic) sample (Figure 4.2):

- No zone (growth completely up to the disc, no clear zone of inhibition)
- Numerous (multiple discrete colonies up to the disc, but within a clear zone of inhibition)
- Hazy, (indistinct – growth within an identifiable zone of inhibition, but no discrete colonies)
- No growth (no colonies – clear zone of inhibition around the disc)

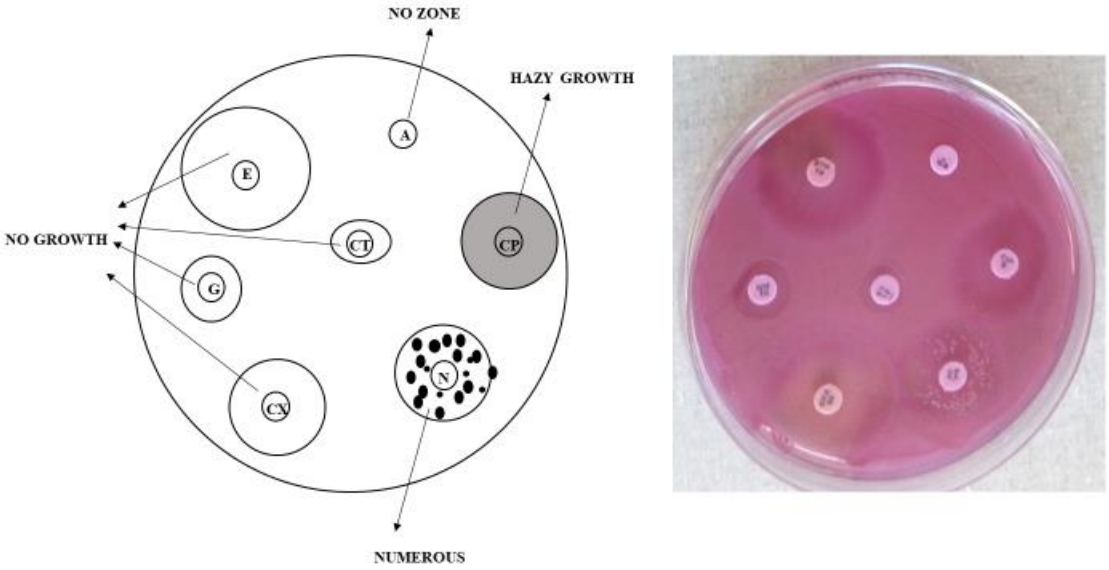


Figure 4.2: Categories used to describe growth around the antibiotic discs, **A**= Amoxicillin, **E**= Ertapenem, **G**= Gentamicin, **CX**= Cefotaxime, **CT**= Colistin, **CP**= Ciprofloxacin, **N**= Nalidixic Acid

The samples exposed to antibiotic were compared to the control and results expressed relative to the control, for each antibiotic disc:

1. Same as control (→)
2. Less than control [fewer colonies around the disc compared to the control sample (↓)]
3. More than control [more colonies around the disc compared to the control sample (↑)]
4. No zone [only applicable if there had been a zone around the disc with the control (↑↑↑)]
5. No growth [no growth of Gram-negative organisms around the disc (↓↓↓)]

A single colony from around each disc (if present) was sub cultured on TBA plates (NHLS Media Laboratory, Greenpoint, South Africa) and incubated as previously described (chapter 2, section 2.2.4). Single isolates from the TBA plate were stored in microbank beads for further investigation.

4.3 Results

The results after exposure to the high concentrations of antibiotics were similar to those after low concentration exposure, as summarized below.

Amoxicillin

There was no notable difference between the susceptibility profiles of isolates from samples after amoxicillin exposure compared to the no antibiotic control (NAC) (Table 4.1), apart from sample D002804J. This sample showed more growth around all of the antibiotic discs (except the quinolone and ertapenem discs) after exposure to both low and high concentrations of amoxicillin.

Ciprofloxacin

Exposure to ciprofloxacin selected for higher rates of resistance to quinolones in nine of the ten samples which were involved in this *in-vitro* pilot study. Eight out 10 samples which had isolates which were resistant to quinolones in NAC developed higher numbers of isolates resistant to quinolones and one sample (D003205H) where no quinolone resistant isolates were previously identified show selection of quinolone resistance (based on the semi-quantitative assessment of growth around the nalidixic acid and ciprofloxacin discs). A smaller number of samples exposed to ciprofloxacin showed increased rates of resistance to amoxicillin (2 samples) and gentamicin and cefotaxime (1 sample; 000805C – after exposure to the high ciprofloxacin concentration) (Table 4.2).

Colistin

Exposure to colistin selected for resistance around the amoxicillin disc in two samples. Decreased growth was also seen around the amoxicillin disc in two samples exposed to colistin. Growth around the quinolone and cefotaxime discs decreased in 7 and 2 samples respectively. However, in one of the samples (D003205H) growth around the colistin disc decreased after exposure to 2ug/mL of colistin and increased after exposure to 0.5 ug/mL of colistin.

Table 4.1: Quantification of growth of isolates within the zone of inhibition of selected antibiotic discs after amoxicillin exposure.

Amoxicillin Exposure										
Concentration	**D003205H**	**D002804J**	**D003206P**	**D003302S**	**D003502A**	**D000706E**	**D003802**	**D003402**	**D004002X**	**D000805C**
	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin
NAC	Numerous	Numerous	No Zone	Hazy Growth	No Zone	No Zone	No Zone	No Zone	Hazy Growth	Hazy Growth
8µg/ml	→	↑↑↑	→	→	→	→	→	→	→	→
2µg/ml	↓	↑↑↑	→	→	→	→	→	→	→	→
	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones
NAC	No Growth	Numerous	Hazy Growth	Numerous	Hazy Growth	Hazy Growth	Numerous	Numerous	Hazy Growth	Hazy Growth
8µg/ml	→	↓	→	→	→	→	→	→	→	→
2µg/ml	→	→	→	→	→	→	→	↑	→	→
	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin
NAC	No Growth	No Growth	No Growth	No Growth	Hazy Growth	No Growth	No Growth	Numerous	Hazy Growth	Hazy Growth
8µg/ml	→	↑	→	→	→	→	→	↓	→	→
2µg/ml	→	↑	→	→	→	→	→	→	→	→
	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime
NAC	1 Colony	No Growth	1 Colony	No Growth	Hazy Growth	No Growth	No Growth	Hazy Growth	Hazy Growth	Hazy Growth
8µg/ml	→	↑	→	→	→	→	→	→	→	→
2µg/ml	↑	↑	→	→	→	→	→	→	→	→
	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin
NAC	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
8µg/ml	↑	↑	→	→	→	→	→	→	→	→
2µg/ml	→	↑	→	→	→	→	→	→	→	→
	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem
NAC	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
8µg/ml	→	→	→	→	→	→	→	→	→	→
2µg/ml	→	→	→	→	→	→	→	→	→	→

NAC=no antibiotic control, →Growth similar to NAC, ↑Increased growth compared to NAC, ↓less growth than NAC, ↑↑↑No Zone, ↓↓↓No Growth, NAC=no antibiotic control, Quinolones= nalidixic acid & ciprofloxacin, NG= No growth on plate. The first column shows the concentration of the antibiotic used. Antibiotics listed in the shaded horizontal rows refer to the antibiotic discs used.

Table 4.2: Quantification of growth of isolates within the zone of inhibition of selected antibiotic discs after ciprofloxacin exposure.

Ciprofloxacin Exposures										
Concentration	**D003205H**	**D002804J**	**D003206P**	**D003302S**	**D003502A**	**D000706E**	**D003802**	**D003402**	**D004002X**	**D000805C**
	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin
NAC	Numerous	Numerous	No Zone	Hazy Growth	No Zone	No Zone	No Zone	No Zone	Hazy Growth	Hazy Growth
1µg/ml	→	NG	→	↑↑↑	↓	↓↓↓	→	→	↑↑↑	→
0.25µg/ml	→	↑↑↑	→	↑↑↑	↓	↓↓↓	→	→	↑↑↑	→
	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones
NAC	No Growth	Numerous	Hazy Growth	Numerous	Hazy Growth	Hazy Growth	Numerous	Numerous	Hazy Growth	Hazy Growth
1µg/ml	↑↑↑	NG	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↓
0.25µg/ml	↑↑↑	↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	→	↑↑↑	↑↑↑	→
	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin
NAC	No Growth	No Growth	No Growth	No Growth	Hazy Growth	No Growth	No Growth	Numerous	Hazy Growth	Hazy Growth
1µg/ml	→	NG	→	→	↓	→	→	↓↓↓	→	↑↑↑
0.25µg/ml	→	→	→	→	↓	→	→	↓↓↓	→	→
	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime
NAC	1 Colony	No Growth	1 Colony	No Growth	Hazy Growth	No Growth	No Growth	Hazy Growth	Hazy Growth	Hazy Growth
1µg/ml	→	NG	→	→	↓	→	→	→	→	↑↑↑
0.25µg/ml	→	→	→	→	↓	→	→	→	→	→
	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin
NAC	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
1µg/ml	→	NG	→	→	→	→	→	→	→	→
0.25µg/ml	→	→	→	→	→	→	→	→	→	→
	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem
NAC	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
1µg/ml	→	NG	→	→	→	→	→	→	→	→
0.25µg/ml	→	→	→	→	→	→	→	→	→	→

NAC=no antibiotic control, →Growth similar to NAC, ↑Increased growth compared to NAC, ↓less growth than NAC, ↑↑↑No Zone, ↓↓↓No Growth, NAC=no antibiotic control, Quinolones= nalidixic acid & ciprofloxacin, NG= No growth on plate. The first column shows the concentration of the antibiotic used. Antibiotics listed in the shaded horizontal rows refer to the antibiotic discs used.

Table 4.3: Quantification of growth of isolates within the zone of inhibition of selected antibiotic discs after colistin exposure.

Colistin Exposure										
Concentration	**D003205H**	**D002804J**	**D003206P**	**D003302S**	**D003502A**	**D000706E**	**D003802**	**D003402**	**D004002X**	**D000805C**
	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin	Amoxicillin
NAC	Numerous	Numerous	No Zone	Hazy Growth	No Zone	No Zone	No Zone	No Zone	Hazy Growth	Hazy Growth
2µg/ml	↓	↑↑↑	→	↓	→	→	→	→	↑↑↑	→
0.5µg/ml	↓	↑↑↑	→	→	→	→	→	→	↑↑↑	→
	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones
NAC	No Growth	Numerous	Hazy Growth	Numerous	Hazy Growth	Hazy Growth	Numerous	Numerous	Hazy Growth	Hazy Growth
2µg/ml	→	↓↓↓	↓	↓	↓	↓↓↓	↓	↓	→	↓
0.5µg/ml	→	→	↓↓↓	→	↓	→	→	→	→	→
	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin	Gentamicin
NAC	No Growth	No Growth	No Growth	No Growth	Hazy Growth	No Growth	No Growth	Numerous	Hazy Growth	Hazy Growth
2µg/ml	→	→	→	→	↓	→	→	↓	→	→
0.5µg/ml	→	→	→	→	→	→	→	→	→	→
	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime	Cefotaxime
NAC	1 Colony	No Growth	1 Colony	No Growth	Hazy Growth	No Growth	No Growth	Hazy Growth	Hazy Growth	Hazy Growth
2µg/ml	→	↓	→	→	↓	→	→	→	→	→
0.5µg/ml	→	→	→	→	→	→	→	→	→	→
	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin	Colistin
NAC	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
2µg/ml	↓	→	→	→	→	→	→	→	→	→
0.5µg/ml	↑	→	→	→	→	→	→	→	→	→
	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem	Ertapenem
NAC	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
2µg/ml	→	→	→	→	→	→	→	→	→	→
0.5µg/ml	→	→	→	→	→	→	→	→	→	→

NAC=no antibiotic control, →Growth similar to NAC, ↑Increased growth compared to NAC, ↓less growth than NAC, ↑↑↑No Zone, ↓↓↓No Growth, NAC=no antibiotic control, Quinolones= nalidixic acid & ciprofloxacin, NG= No growth on plate. The first column shows the concentration of the antibiotic used. Antibiotics listed in the shaded horizontal rows refer to the antibiotic discs used.

4.4 Discussion

The effect of varying (and sometimes sublethal) antibiotic concentrations on the selection of resistant bacteria is unclear (Gullberg *et al.*, 2011). The gut flora of humans and animals are frequently exposed to a variety of concentrations of antibiotics due to their widespread use. In many instances, the concentrations in the gastrointestinal tract are sublethal and may select for resistance (Andersson & Hughes, 2014). This chapter set out to study the effects of low-level antibiotic exposure on the development of resistance in stool isolates, using a relatively simple *in-vitro* model. While the effects were modest, and no assessment was made of resistance mechanisms, there was some evidence that in a proportion of samples, greater numbers of resistant organisms were present after antibiotic exposure and notably, resistance to agents other than that to which the sample had been exposed.

A previous study conducted by Kohanski *et al*, (2010) showed that when a wild-type *E. coli* was exposed to ampicillin (1µg/ml), the ampicillin MIC in the exposed isolates was 5-fold higher than the unexposed. In addition, isolates from the ampicillin exposure developed resistance to norfloxacin and kanamycin. However, in our study, the amoxicillin exposures did not select for higher levels of resistance except for one sample (D002804J), which is most likely because most of the isolates were resistant to amoxicillin prior to the exposures.

In another study, *E. coli* isolates were exposed to sub-inhibitory concentrations of ciprofloxacin (0.1 x MIC) (Gullberg *et al.*, 2011). This led to higher resistance, and even after several generations, *E. coli* isolates had a ciprofloxacin MIC that was 2-fold higher compared to wild-type isolates. Similarly, in our *in-vitro* study, exposure to ciprofloxacin selected for high rates of resistance to ciprofloxacin. The only sample included in this pilot study which had no quinolone-resistant isolates at baseline (D003205H), showed the presence of quinolone resistant isolates upon exposure to ciprofloxacin. Samples which had isolates resistant to only nalidixic acid in the no antibiotic control, upon exposure to ciprofloxacin yielded ciprofloxacin-resistant isolates as well. Although quinolone MICs were not determined, samples with isolates which were resistant to nalidixic acid and ciprofloxacin but with measurable zones around the discs, upon exposure to ciprofloxacin had no zones around the two discs, suggesting an increase in the MIC, or an

increase in the number of resistant isolates (or both). Our results are similar to those of an *in vivo* study, which showed the emergence of resistance when commensal bacteria were exposed to sub-MIC concentrations of ciprofloxacin (Fantin *et al.*, 2009). Healthy adults were given a daily dose of ciprofloxacin (250 mg twice a day or 500 mg twice a day) and fecal samples were collected prior to treatment and on days 7, 14, and 42. The concentration of ciprofloxacin in the stool was determined using liquid chromatography, and ciprofloxacin was not detected in the stool at day 42. Resistance emerged in 25% of the participants mainly when the concentration of ciprofloxacin was sub MIC (Fantin *et al.*, 2009). The emergence of resistance was however not significantly different across the different antibiotic dosages, which is similar to our findings where both concentrations of ciprofloxacin resulted in increased numbers of resistant isolates.

Exposure to colistin demonstrates an irregular selection which could not be followed up. Analysis of our results is likely confounded by the poor performance of colistin discs on agar plates. For this reason, disc diffusion has not been approved for colistin susceptibility testing. Nine out of 10 samples showed no difference compared to the NAC around the colistin disc, and six out of 10 samples showed no difference between the growth around the various discs in the colistin exposures and the NAC. Four of the samples differed around other antibiotic discs, but this did not follow any specific pattern.

However, when sample D002804J was exposed to 1 µg/ml of ciprofloxacin there was no growth on the plate. This could mean that the higher concentration of ciprofloxacin eliminated all the isolates in the sample.

One other thing is to consider is whether sub-MIC antibiotic concentrations in the environment are able to select and enrich resistance as we have shown in our study. When patients are exposed to low doses of antibiotics for a long period of time, or animals are treated with low antibiotic concentrations to promote growth, a concentration gradient is built in the various compartments of the body; especially the intestines. These concentrations are likely to be at sub-MIC levels which can select for resistance. The environment might be serving as a major reservoir because antibiotic use is not controlled in community settings, and in many countries (especially low- and middle-income countries) people can buy antibiotics over the counter.

These antibiotics may be of poor quality with reduced activity and combined with people either not completing the treatment course or taking doses erratically, may expose microorganisms to low-level antibiotic concentrations. Patients treated with antibiotics may excrete these into the sewage system, which is then discharged into water bodies in the environment. Fluoroquinolones are known to reach high levels in hospital sewage (Larsson *et al.*, 2007; Lien *et al.*, 2016; Picão *et al.*, 2013).

More quinolone resistant isolates were detected in the NAC than using the original baseline screening approach; and therefore, the baseline stools should be screened using multiple different antibiotic discs for the detection of isolates resistant to all classes of antibiotics. Another approach which can be used for screening for resistance is a direct/targeted metagenomics study, which could be considered for further studies. Direct/targeted metagenomics targets all organisms in a microbial community (resistome) and does not focus on a specific genus/family as we did in our study.

The major limitation of the study is that the duration of exposure was short, and the quantification process was relatively crude. It is also important to note that the *in-vitro* study tries to simulate what happens in the gut, but we don't know what role, if any, the interaction between the microbes in the stool samples plays in the development of resistance.

We did not determine the MICs of the isolates at baseline and after exposure but with reference to the ciprofloxacin exposures we can say that sublethal antibiotic concentrations are likely to enrich and select for *de novo* resistance. The determination of MIC at baseline and after exposures is vital because it will provide the clinical significance of the sublethal antibiotic exposures.

This approach worked in principle, nevertheless the *in-vitro* model is crude, and a more sophisticated model needs to be developed to understand the effect of *in-vitro* antibiotic exposure. The use of a wider class of antibiotics should be considered for further studies since it will help understand the role of sublethal antibiotic concentrations in the selection of resistance.

Chapter 5 : General Discussion

Antibiotic resistance is a global challenge, especially in low- and middle-income countries. Various interventions have been proposed to help estimate the impact of antibiotic resistance, including public education, surveillance and understanding the epidemiology of resistance (WHO, 2014). Not enough has been done in developing countries to implement policies to mitigate antibiotic resistance and control antibiotic usage (Laxminarayan & Heymann, 2012). However, for the past six years South Africa has implemented major policies to mitigate antibiotic consumption. This includes the establishment of the South African Antibiotic Stewardship program (Schellack *et al.*, 2017).

Antibiotic surveillance is needed to combat antibiotic resistance because it will provide the data required for the development of strategies and actions to mitigate antibiotic resistance (CDC, 2013). For antibiotic surveillance to be effective, population-based surveillance needs to be encouraged (WHO, 2014). The TB-CHAMP trial provided an opportunity to obtain data on resistance rates in a community-based, pediatric population. We aimed to describe baseline rates of antibiotic resistance in Gram-negative bacilli (*E. coli* and *Klebsiella* spp.) in children in Cape Town and to investigate the effect of antibiotic exposure on the development of resistance in stool samples.

Fifty participants were included in this study and 66% of them were colonized by isolates resistant to at least one antibiotic. More concerning was that 34% were colonized by MDR isolates. A single *Klebsiella pneumoniae* isolate was resistant to carbapenems which is unexpected in the community. Carbapenems are last resort antibiotics and the production of carbapenemases are a major cause of carbapenem resistance (Papp-Wallace *et al.*, 2011). The carbapenem-resistant isolate harbored the NDM gene which is the most common carbapenemase gene reported in *K. pneumoniae* isolates in South Africa. To the best of our knowledge, this is the first study to show carriage of carbapenem resistant isolates in otherwise healthy children in a community setting in South Africa. Carbapenem-resistant Enterobacteriaceae (CRE) are an emerging health risk and further community surveillance needs to be done to better understand and estimate the prevalence of CRE in the community.

Resistance to third generation cephalosporins is a major indicator of ESBL production (Paterson & Bonomo, 2005), and 34% of the participants were colonized by ESBL-producing isolates based on PCR and sequencing. The only ESBL gene was CTX-M, which is the most common ESBL gene reported in community and nosocomial settings (Cantón & Coque, 2006). While genes such as SHV-1, SHV-11, and SHV-26 were also identified, these genes are considered non-ESBLs because they lack extended spectrum activity. The high rate of ESBL carriage in our study is concerning since these enzymes are plasmid-encoded and are likely to be transferred among different bacterial species. These enzymes are also a global threat because they confer resistance to the cephalosporin class of antimicrobials, which are commonly used agents worldwide. Global usage of antibiotics increased by 36% from 2000-2010 and in 2010 cephalosporins and extended-spectrum penicillins accounted for 55% of the total antibiotics consumed (Van Boeckel *et al.*, 2014)

Quinolones are another commonly used class of antibiotics (Aldred *et al.*, 2014). While resistance to quinolones has typically been associated with mutations in the *gyrA* and *parC* genes, plasmid-mediated quinolone resistance genes such as *qnrA*, *qnrB* and *qnrS* have also been associated with quinolone resistance. All isolates resistant to quinolones were subjected to PCR for the identification of plasmid-mediated quinolone resistance genes and for the detection of mutations in the *gyrA* and *parC* genes. The most common quinolone resistance mechanisms in our isolates were mutations in the *parC* and *gyrA* genes, which are not transferable but lead to higher levels quinolone resistance. Mutations in *gyrA* or *parC* were detected in all isolates with quinolone resistance. Four out of 5 *E. coli* isolates harboring *qnrS* also had double mutations in *gyrA* and a single mutation in *parC*. K4 (the carbapenem-resistant isolate) was the only *Klebsiella* isolate with mutation in *gyrA* and a triple mutation in *parC;* all the others had double mutations in *parC* and no mutation in *gyrA*.

The commonest plasmid-mediated quinolone resistance gene was *qnrB* in *Klebsiella* isolates and *qnrS* in *E. coli* isolates. Contradicting our findings, a study in healthy children in Peru and Bolivia revealed that the majority of *E. coli* carried *qnrB* while *Klebsiella* isolates carried *qnrS* (Chapter 1, section 1.5.1.2). However, the presence of one type of plasmid mediated quinolone resistance in *E. coli* and a different one in *Klebsiella* isolates is novel and unexplained. Our

findings reveal a high rate of quinolone-resistance in our isolates, raising the question of what might be driving the high prevalence of quinolone resistance in the community. All mutations seen in quinolone-resistant *E. coli* and *Klebsiella* isolates have been commonly reported in literature to cause quinolone resistance (Nouri *et al.*, 2016; Onseedaeng & Ratthawongjirakul, 2016). MIC testing will help to understand how the number of mutations influences the selection of the appropriate dosage of quinolones.

To study the acquisition of resistance, an *in vitro* model was employed. The stool samples were exposed to varying concentrations (low concentrations are potentially more likely to select for resistance) of amoxicillin, ciprofloxacin, and colistin. Although most of our samples had amoxicillin-resistant isolates at baseline, the only sample which had an amoxicillin-susceptible isolate develop amoxicillin resistance when exposed to amoxicillin. All samples exposed to ciprofloxacin either generated ciprofloxacin resistant isolates (if not detected previously) or increased the proportion of resistant isolates (if resistance present prior to ciprofloxacin exposure). This in itself may not be particularly surprising, as exposure to ciprofloxacin might be seen as a screening process to enhance the recovery of resistant isolates. However, further studies investigating the effect of low-level exposure on the spread of mobile elements and the rate of selection may provide important insights into the mechanisms driving resistance selection. The use of levofloxacin for MDR-TB prophylaxis in the TB-CHAMP project is likely to select for resistance based on what we have seen in our *in vitro* model. This may be aggravated by the fact that quinolones have a high recovery rate in stools (De Lastours *et al.*, 2014).
Colistin exposures did not select for higher resistance around the colistin discs but rather reduced the growth around the various antibiotic discs.

The clonal relatedness of isolates in the stool of the participants was greatly diverse which made it difficult to test the hypotheses related to the role of bacterial lineage on the development of resistance (Jayol *et al.*, 2014). Future studies could focus on *in-vitro* exposure using different lineages, this might be a way to assess whether certain lineages acquire resistance more readily than others.

Our results suggest that resistant *E. coli* and *Klebsiella* spp are frequently carried in the stools of children in Cape Town communities and pose a risk to empiric treatment practices. This supports the speculation that resistance may reflect "spillover" from hospital settings. To confirm this speculation, further studies should be done by screening patients before admission and after discharge to determine whether they have acquired resistant isolate while hospitalised, which they might be carrying into the community. The presence of the plasmid-mediated genes is concerning and may result in increased quinolone and cephalosporin resistance. In conclusion, we suggest that a larger sample size and a metagenomic study should be employed in further studies to investigate the burden of antibiotic resistance carriage in the community. Recruiting a larger portion of the population will help understand the mode of trasmission of antibiotic resistance whiles the metagenomic study allows one to assess both cultured, uncultured bacteria and a broader range of resistance genes. It also helps in discovering novel resistance genes.

Appendices

Appendix 1: Stool SOP TB-CHAMP Version 1_ 20190114

	DESMOND TUTU TB CENTRE (DTTC) **STANDARD OPERATING PROCEDURE (SOP)**
Division:	
SOP Number:	
SOP Name:	Study Specific stool collection aspects for TB CHAMP
SOP version:	Version 1.0
SOP version date:	14 January 2019
Training Implications:	

Principal Investigator	☒	Regulatory	☒
Sub-Investigator	☒	Data Personnel	☐
Study Coordinator	☒	Finance Personnel	☐
Pharmacist	☐	Receptionist/Admin personnel	☐
Study Nurse	☒	Driver	☒
Lab Personnel	☐	Cleaner	☐
Counsellor/Recruiter	☒	Other	☐

SOP Compiled By:	Kristien Nel Van Zyl (Division Medical Microbiology, SU); Supervisor: Prof A Whitelaw
SOP Approved by:	_____ _____ Prof Anneke Hesseling Date

FOR REGULATORY USE ONLY			
Distributed to:			
Site:	**Name & Surname:**	**Signature:**	**Date:**
Tygerberg			
Brooklyn Chest Hospital			
Ubunye			
Beautiful Gate			
Tambo Road			
Kuilsriver			
Data Office			
Lab Office			

SOP Review and version management			
Review Dates	Reviewed By	Changes Required	Version & Date

STANDARD OPERATING PROCEDURE (SOP) - Study Specific stool collection aspects for TB CHAMP.

Objectives

This SOP describes the procedure for collection of stool specimens for the purpose of bacterial culture and molecular microbiome studies, in the context of the TB-CHAMP study.

Procedures

Equipment

Table 1: Equipment required

STOOL COLLECTION	
1	Sample container. Faecal Container (screwcap) With Spoon (25 ml) (LASEC product number: PLPS109047)
2	Leak-proof bag
3	Opaque carrier to protect dignity
4	Cling-wrap (optional)
5	Potty/Bed-pan (optional)
TRANSPORT	
6	Labels and indelible marker pen
7	Transport coolbox
8	Icebrick
9	Stool requisition form
SAMPLE PROCESSING	
10	Cotton Swabs
11	Cary Blair Medium
12	DNA buffer (if used) or -80 freezer

Stool collection

1. **Procedure for clinic collection**
   1.1. Study nurse/ counsellor must collect stool as per DTTC stool collection SOP (PC011 Stool Collection Version 1.0)
   1.2. Make sure no urine, water, soil or other material gets in the container. An attempt should be made to collect a specimen that consists primarily of stool when only a mixture of stool and urine is available. The stool should be mixed with the spoon at least five times to generate a homogeneous mixture. At least 6 scoops of stool needs to be added into the container. If the stool is loose, collect at least 3 scoops of watery stool (only if it is free from urine as stated above) and 3 of more solid stool.
   1.3. Ensure that the specimen containers are sealed well and disinfect the outside with 70% alcohol if necessary. The container should be placed in a sealable plastic bag. Label the collection container with the Subject Event barcode.
   1.4. The clinician / Study nurse / counsellor should complete the stool requisition form (DTTC TB-CHAMP Stool Requisition Form V1.2
   1.5. Keep specimen in the fridge (2-8°C) at the site and transport to Tygerberg Hospital in coolboxes on ice on the same day as collection, or as soon as possible. Avoid freezing the sample as subsequent thawing during transport may cause DNA degradation.
2. **Procedure for home collection**
   2.1. Clear and simple verbal instructions regarding the method of stool collection should be given to the caregiver by the study nurse/ counsellor or counsellor, along with the instruction leaflet (TB-CHAMP_Parent Instruction Leaflet_V1.0).
   2.2. The caregiver should be given a stool container, leak-proof bag and opaque plastic box
      - In addition, if the caregiver has a freezer at home, a coolbox and icebrick should be provided. The icebrick must be kept in the freezer until required (see 2.4)
   2.3. Encourage the caregiver to put the stool sample in the collection container provided by the study team, and to note the time and date of stool collection - preferably taken as soon as possible before the clinic visit (night before, or ideally the morning of the clinic visit).
   2.4. Once collected, the sample should be kept on the icebrick in a coolbox (if provided), or in the coolest place in the home until delivered to clinic.
      - The study team must inform the caregivers that stool should not be collected on a Friday afternoon as the sample will only reach DTTC on the Monday.
   2.5. For baseline (BL) stool should be taken at screening visit, and if not obtained a sample collection kit will be provided for home collection. The kit and sample will then be returned at BL visit.

- In cases where screening and BL occur on the same day, the stool should preferably be collected before treatment/placebo is issued. If this is not possible, then the stool can be collected within 24 hours of the treatment/placebo.
- For 8-, 16-, 24- and 48-week follow-up samples, if participants are unable to deliver a stool sample at the clinic, a sample collection kit will be provided to them for home collection. A driver will pick-up the samples from the home. Stool samples can be collected up to 2 weeks after the visit.
- The study team should follow-up with the caregivers everyday after the visit until they can confirm that a sample was taken and collected.
- No suppository should be given to the child for the purpose of the stool collection.

Transport

1. When a driver collects a stool sample (from clinic OR participant home), he / she should
   1.1. Have a cooler box and frozen ice brick ready for transporting stool samples
   1.2. Wear gloves, ensure that the collection container is sealed and disinfect the outside with 70% alcohol if necessary
   1.3. Fill out the stool requisition form (if not completed by clinician / study nurse/ counsellor (1.4) and ensure the Subject Event barcode is pasted on the collection container.
2. Preparation of specimen and transport to the Laboratory
   2.1. The specimen must be transported to the Division of Medical Microbiology (Room 561, 9th Floor, Green Lane East, Tygerberg Hospital) as soon as possible in a cooler box with an ice pack to keep it at 2-8°C during transport.
   2.2. If delivery to TBH is unsuccessful, the sample should be brought to the DTTC (Room 0065, K-floor, Stellenbosch University Medical School) and stored at -80°C. This should also be noted in the comment section of the requisition form.
   2.3. A WhatsApp group called “TB-CHAMP Stool” will be created to enable communication between the Medical Microbiology laboratory team members, at least one member from the DTTC laboratory and the drivers.

Lab Section Only: Sample processing

1. Upon arrival at the Medical Microbiology laboratory, the stool requisition form will be signed by the recipient, noting the time and date of arrival.
2. The sample will be split into two as soon as possible
   2.1. A swab or spoonful of stool is to be transferred to Cary Blair medium for culture work.
   2.2. A spoonful of stool is to be transferred to DNA stabilizing buffer (if used)
   2.3. The remainder of the sample will be frozen and stored at -80°C

2.4. A separate sample barcode will be created for each sample split as outlined above and placed on the requisition form.

QC Process for Stool Requisition form

The final QC of the stool requisition form will be performed by the Medical Microbiology TB-CHAMP personnel. Should there be any missing fields or incorrect data on the stool requisition form, the Medical Microbiology TB CHAMP personnel will follow the below steps to rectify the error.

a. Complete the Data Clarification Form (DCF) for stool Collected V1.0. Indicate PID and current data that needs to be rectified.
b. Email the Data Clarification form to the site contact;
    i. Contact at Beautiful Gate: Andiswa Vavi: bpvavi@sun.ac.za, 083 774 2527
    ii. Tambo Road: Daphne van Ster: daphnev@sun.ac.za, 0834839075
c. Site will complete and sign Data Clarification form and email the form back to the Medical Microbiology TB CHAMP staff.
d. Medical Microbiology TB-CHAMP personnel will then be print and staple DCF to the original stool requisition form.

	STANDARD OPERATING PROCEDURE (SOP) **SIGNATURE LOG**
Division:	
SOP Number:	
SOP Name:	Study Specific stool collection aspects for TB CHAMP
SOP version:	Version 1.0
SOP version date:	14 January 2019

NAME	**SIGNATURE**	**DATE**

Appendix 2: DTTC TB-CHAMP Stool Requisition Form V1.2 20190206

DTTC TB-CHAMP Clinical Trial

REQ1 – Stool Requisition Form

PID

Collection Date	D	D	M	M	Y	Y	Y	Y
Collection Time	H	H	H	M	M	M		

Pick-up Date	D	D	M	M	Y	Y	Y	Y
Pick-up Time	H	H	H	M	M	M		
Drop-off Time	H	H	H	M	M	M		

Visit	0	0_Baseline
	1	1_8 weeks
	2	2_16 weeks
	3	3_24 weeks
	4	4_48 weeks

Collection method	1	1_Stool collected from diaper
	2	2_Stool collected from cling film over toilet
	3	3_Stool collected from potty/bedpan

Site	1	Tambo Road
	2	Beautiful Gate
	3	7th Floor Tygerberg
	4	Home
	5	Robert Gie Ikamva Wing

Storage method	1	1_Stool stored in fridge
	2	2_Stool stored in coolbox with icebrick
	3	3_Stool stored without icebrick

Comment:

FOLLOWING SECTION FOR LAB USE ONLY: STOOL SAMPLES ONLY

Sample Barcode #1	Sample Barcode #2 (Culture)	Sample Barcode #3 (Microbiome)

	Stool Processed		Stool Processed		Stool Processed
	Stool Stored		Stool Stored		Stool Stored

Stool weight processed	g	Stool Appearance		Solid		Liquid
Processed by:				Semi solid		Sticky
Processed date:	D D M M Y Y Y Y					

Comment (stools):

FOLLOWING SECTION TO BE COMPLETED BY DRIVER/CLINICIAN AND TBH RECIPIENTS

	Initial	Date								Signature
Form Completed by:		D	D	M	M	Y	Y	Y	Y	
Site QC completed by:		D	D	M	M	Y	Y	Y	Y	
Lab QC completed by:		D	D	M	M	Y	Y	Y	Y	
Form Captured by:		D	D	M	M	Y	Y	Y	Y	

Appendix 3: TB-CHAMP_Caregiver Instruction Leaflet Nappies_20180528

GOAL: We look at stool to find out how germs in the gut are changed by the medicine. This will help us decide about using this medicine in future.

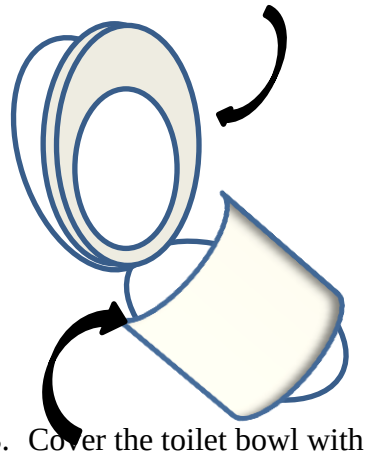
Tips for collecting a stool sample from toilet-trained children

- Many older children do not like to give a sample of their stool for testing. Talk to your child using simple, honest words. Show them you understand by saying something like "I know this may be embarrassing for you, but we need to do this because…" This will allow your child to express their feelings and accept your help.
- When taking the stool sample, make sure that the sample does not fall into the toilet.
- If the stool falls into the toilet water or is mixed with urine, **do not** collect it.
- Watch your child if they are young and might not understand that their urine must be kept separate from the stool being collected.

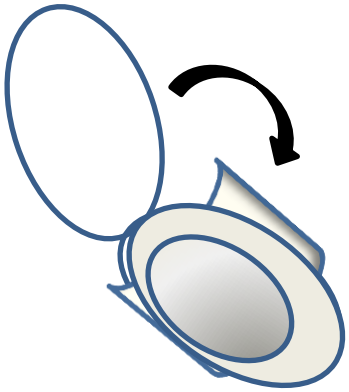
You can collect the stool like in **option 1** (below) **or option 2** (on back of page):

Option 1: You can collect a stool sample by stretching plastic wrap/sheet over the toilet

A. Lift the toilet seat



B. Cover the toilet bowl with plastic sheet



C. Lower toilet seat over the plastic

Follow these steps:

a. First have your child pass urine into the toilet, without pooping.

b. Lift the toilet seat and cover the entire bowl with a large sheet of plastic. If the plastic sheet does not stick to the toilet, you may need to use tape to stick it down.

c. Make a small dip in the middle of the plastic so there is a place for the stool to collect.

d. Lower the toilet seat cover and have your child poop onto the plastic sheet.

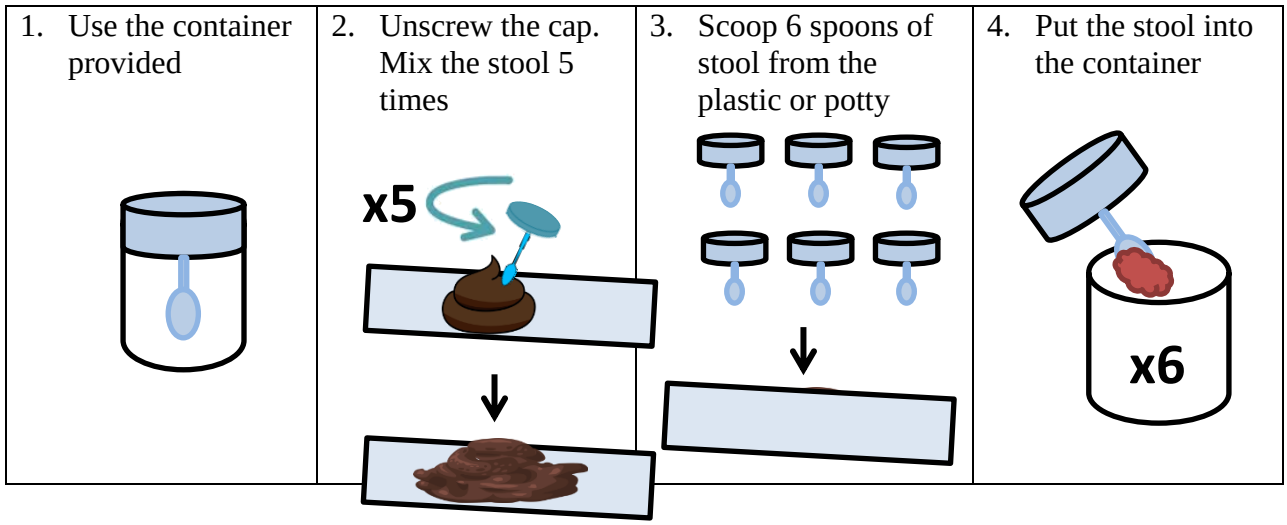
e. Using the scoop or the small white spoon given to you, transfer the last part of the stool into the collection container given to you. Collect at least 6 scoops into the container. Follow the steps on the next page.

Option 2; You can collect a stool sample using a potty.

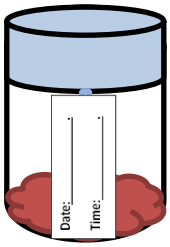
Follow these steps:

a. Let the child first pass urine into the toilet.
b. Then have the child sit on the potty and pass stool.
c. Using the scoop or the small white spoon given to you, transfer the last part of the stool into the collection container given to you. Collect at least 6 scoops into the container and fill it up to the mark. Follow the method shown below.

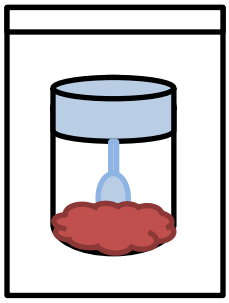
Steps for collecting stool from the plastic sheet or potty into the container:



5. Screw the cap tightly closed. Write down the date and time on the label on the container

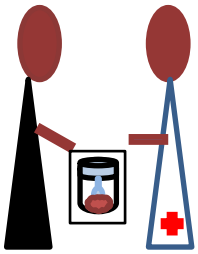


6. Put the container inside the plastic bag provided and seal the bag

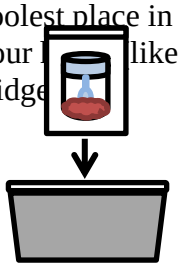


7. Wash hands very well with soap and water

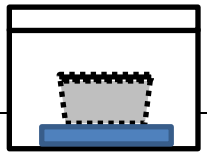
8. If you are at the clinic let the study nurse know as soon as possible that you have collected the stool sample



9. If you are at home, put the bag with the container in the box provided and keep in the coolest place in your [illegible] like a fridge



10. If you were provided with a cooler box and ice brick, put the lunchbox in the cooler box on the frozen icebrick when you bring it to the clinic



NOTE:

- If the stool is watery, try not to let the stool run off the plastic.
- Collect at least 3 scoops of watery stool and 3 of more solid stool.
- If it is all watery, collect 6 scoops of watery stool.
- It is important that no urine or toilet water mix with the stool sample.

Appendix 4: Biomarkers

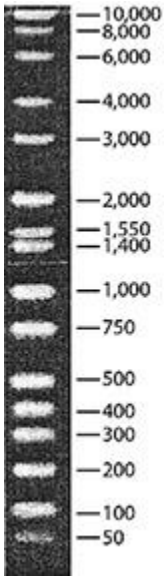


Figure 5: DirectLoad™ Wide Range DNA Marker (Sigma, Aldrich, Germany)

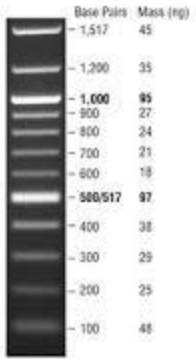


Figure 6: New Biolabs 100bp Ladder (New England Biolabs Inc.).

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